"The Role of Endothelins in Ovarian and Breast Cancer"

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Στους γονεις μου, Αγγελικη και Γιωργο, και στην Ελλας.

DECLARATION

In accordance with the regulations of the University of Edinburgh, I declare that the material described in this thesis is composed by myself entirely, except from where acknowledgement has been made in the text.

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ABSTRACT

The endothelin (ET) family consists of three structurally-related peptides (ET-1, ET-2 and ET-3) which interact with two populations of receptors (ET_A-R and ET_B-R). ETs and their receptors are expressed by a variety of tissues in which they may be involved in a multiplicity of functions including regulation of mitogenesis. ETs may also be expressed by cancer cell lines and may be present in tumours at concentrations much higher than those in normal tissues. Despite this, the involvement of ETs in cancer is largely undefined.

The aim of this project was to investigate the potential involvement of endothelins in the breast and ovarian cancer by (i) measuring the expression and secretion of endothelin by epithelial and fibroblast cell lines derived from ovarian and breast cancers, (ii) determining the expression of specific receptors for endothelins in the same cell lines, (iii) monitoring the effects of exogenous additions of endothelins on the growth of the cell lines in culture, (iv) measuring the expression of endothelins and their receptors in a series of primary ovarian and breast cancers.

To investigate endothelin production by ovarian and breast carcinoma and fibroblast cell lines, ET-1-like material was detected by radioimmunoassay in conditioned media collected from two ovarian (PEO4 and PEO14) and three breast carcinoma (MDA-MB-231, T47D, ZR-75-1) cell lines. Addition of either bombesin or IL-6 to the culture media increased production of immunoreactive ET from the cell lines. In contrast, ET-like material was not found in the conditioned media from three ovarian (PEO12F, PEO14F, PEO27F) and two breast (Sharlow, Dunbar) human fibroblast cell lines. Expression of mRNA for endothelins was investigated using RT-PCR analysis, and by this methodology, mRNA for ET-1 and ET-3 were detected in ovarian and breast carcinoma cell lines. Hybridisation of the RT-PCR products with specific oligonucleotides designed for each ET confirmed their nature. This suggests that the major source of ET in breast and ovarian tumours

is from the epithelial cells rather than the stromal compartment. Immunohistochemical staining of ovarian and breast tumours with an antibody specific for ET-1 indicated the presence of the protein in these tumours. RT-PCR analysis demonstrated not only the presence of ET-1 but also ET-3 mRNA.

ET receptors were measured using a ligand binding assay and expression of ET receptor mRNA was detected by RT-PCR. Binding studies using ^{125}I-ET-1 failed to indicate the presence of specific ET receptors in breast epithelial cancer cell lines but ET receptors were detected in the two ovarian carcinoma cell lines. Scatchard analysis indicated the presence of two binding sites in one ovarian carcinoma cell line (PEO4) and a single binding site in another line (PEO14). Competition experiments using ^{125}I-ET-1 in the presence of increasing concentrations of ET-1, ET-3, an ET_A-R antagonist (BQ123) and an ET_B-R antagonist (BQ788) indicated the expression of specific receptor subtypes in the carcinoma and fibroblast lines. RT-PCR analysis detecting mRNA for ET_A-R and ET_B-R receptor subtypes provided further identification of the receptor subtypes.

The growth of breast carcinoma cell lines was unaffected by the exogenous addition of ETs while ET-1 and ET-2 but not ET-3 addition stimulated the growth of ovarian carcinoma cells. Exogenous addition of ET-1, ET-2 and ET-3 stimulated the growth of both ovarian and breast fibroblast cell lines. The antagonists BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist) inhibited ET-stimulated growth in a manner consistent with the receptor subtypes present in each cell line. In the absence of exogenous ET, the antagonists produced small inhibitory effects of growth in the ovarian carcinoma cell lines. This may be caused by the antagonism of ETs secreted by the same cells and could be consistent with autocrine stimulation of growth by endothelins.

Co-culture of epithelial and fibroblast cells produced increased growth of both cell types relative to that when individual cell types were cultured separately. This indication, that increased growth could be blocked by addition of ET antagonists, suggests possible paracrine regulation of

growth by ETs.

These results support the view that endothelins may affect the growth of ovarian and breast tumours and these influences may operate through autocrine/paracrine loops.

ABBREVIATIONS

aa	- amino acid
AA	- arachidonic acid
ACN	- acetonotrile
Bmax	- maximum binding
Bo	- maximum binding in radioimmunoassay
BSA	- bovine serum albumin
$CHCl_3$	- chlorophorm
℃	- degrees Centigrade
Ca^{2+}	- calcium ion
$CaCl_2$	- calcium chloride
cAMP	- cyclic adenine momophosphate
cGMP	- cyclic guanine monophosphate
Ci	- Curie
cm	- centimetre(s)
CO_2	- carbon dioxide
cpm	- counts per minute
cDNA	-"complementary" deoxyribonucleic acid
DAB	- diaminobenzidine
DAG	- diacylglycerol
DCIS	- ductal carcinoma in situ
DMEM	- Dulbecco's modified Eagle medium
DNA	- deoxyribonucleic acid
dpm	- dissociations per minute
ECE	- endothelin converting enzyme
EGF	- epidermal growth factor
EGFR	- epidermal growth factor receptor
EtOH	- ethanol
ER	- oestrogen receptor
ET-1	- endothelin-1
ET-2	- endothelin-2
ET-3	- endothelin-3

ET_AR	- type A endothelin receptor
ET_BR	- type B endothelin receptor
FCS	- foetal calf serum
FSH	- follicle stimulating hormone
FGF	- fibroblast growth factor
g	- gram(s)/centrifugal field
H_2O	- water
HCl	- hydrochloric acid
HITS	- hydrocortisone, insulin, transferrin, sodium selenite
ICRF	- Imperial Cancer Research Fund
IGF-1	- Insulin-like growth factor type 1
IGF-2	- Insulin-like growth factor type 2
IL-6	- Interleukin-6
IP_3	- inositol trisphosphate
IU	- international unit(s)
kb	- kilobase(s)
KBq	- kilo bequerel(s)
Kd	- affinity of binding
kDa	- kilodalton(s)
LCIS	- lobular carcinoma in situ
µg	- microgram(s)
µl	- microlitre(s)
M	- molar concentration
MeOH	- methanol
mg	- milligram(s)
$MgCl_2$	- magnesium chloride
ml	- millilitre(s)
mRNA	- messenger ribonucleic acid
Mw	- molecular weight
NaCl	- sodium chloride
NaOH	- sodium hydroxide

NMR	- nuclear magnetic resonanse
NSB	- non-specific binding
OD	- optical density
PA	- phosphatidic acid
PBS	- phosphate buffered saline
PCR	- polymerase chain reaction
PDGF	- platelet derived growth factor
PEG	- polyethylene glycol
PG	- prostaglandin
PIP2	- Phosphatidyl Inositol bisphosphate
PKA	- protein kinase A
PKC	- protein kinase C
PLA_2	- phospholipase A_2
PLC	- phospholipase C
PLD	- phospholipase D
PR	- progesterone receptor
PT	- pertusis toxin
RIA	- radioimmunoassay
rpm	- revolutions per minute
RT	- reverse transcriptase
SD	- standard deviation
sec	- second(s)
SRTX	- sarafotoxin
SSC	- saline sodium citrate
TB	- tris buffer
TBS	- tris buffered saline
TCA	- trichloroacetic acid
TDLU	- terminal duct lobular unit
TGF	- tumour growth factor
TFA	- trifluoroacetic acid
TNF	- tumour necrosis factor
w	- weight

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CHAPTER 1: INTRODUCTION

1.1 Introduction

The scope of this study was to investigate the potential role of endothelins in breast and ovarian cancer. The first two section of this introductory chapter are concerned with general features of ovarian and breast cancer. In the third section, the latest information about the structures and biology of endothelins and their receptors is reviewed and what is known so far of their roles in ovarian and breast cancer. Finally, at the end of the chapter, the main aims of this study are described.

1.2 Ovarian cancer

1.2.1 Introduction/epidemiology

Ovarian cancer is a major gynaecological disease being the fifth most common cause of cancer in women. On average one woman in 70 will develop ovarian cancer and almost one in 100 will die from the disease. Ovarian cancer is most common in women of age 70-80 years of age and very rare in women under 45 years of age. The five year survival rate is low at approximately 30% due to the generally advanced stage of disease at diagnosis.

1.2.2 Origins of ovarian cancer

Ovarian cancer presents in a variety of histologically different forms the existence of which can be explained by the range of epithelial tissue types. The latter are generated during embryological development and reflect the physiology of the ovaries (Young et al., 1992). Thus, neoplasms may originate from the epithelial cells of the surface of the ovaries, the germ cells or the stroma of the ovary (Serov and Scully 1973). Epithelial ovarian carcinomas represent more than 90% of the malignant neoplasms of the ovaries. Histological studies have suggested that these types of neoplasms show close resemblance to the Müllerian epithelium which during development has formed the fallopian tubes, the uterine body and the uterine cervix, which are lined with distinctive epithelia. The differentiated epithelium of serous tumours is similar to the

epithelium of the fallopian tubes; that of mucinous tumours similar to the endocervical epithelium and that of endometroid and clear cell tumours, similar to the epithelia of the endometrium. The extent of progression of this disease can be detected by surgery. Thus, the stage of the disease is defined by the extent of the spread which is reported at laparotomy (the stages of the disease as defined by the International federation of gynaecology and obstetrics (FIGO, 1988, Rio de Janeiro) are shown on table 1.1).

Stage	Definition
I	Growth limited to the ovaries
IA	Growth limited to the ovary; no ascites; no tumour on the external surface; capsule intact
IB	Growth limited to both ovaries; no ascites; no tumour on the external surfaces; capsule intact
IC*	Tumour either stage Ia or Ib, but with tumour on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings
II	Growth involving one or both ovaries with pelvic extension
IIA	Extension and/or metastasis to the uterus and/or tubes
IIB	Extension to other pelvic tissues
IIC*	Tumour either stage IIa or IIb but with tumour on surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings
III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes; superficial liver metastases; tumour is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum
IIIA	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
IIIB	Tumour involving one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces none exceeding 2 cm in diameter; nodes are negative
IIIC	Abdominal implants greater than 2 cm in diameter and/or positive retroperitoneal or inguinal nodes
IV	Growth involving one or both ovaries with distant metastases. If pleural effusion present, there must be positive cytology to assign a case to Stage IV; parenchymal liver metastasis

* To assess the impact on prognosis of the different criteria for assigning cases to Stage IC or IIC, it would be of value to know whether the source of malignant cells was (I) peritoneal washings, or (ii) ascites, and whether rupture of the capsule was spontaneous or caused by the surgeon

Table 1.1: FIGO stages for primary carcinoma of the ovary (International Federation of Gynaecology and Obstetrics 1988, Rio de Janeiro).

1.2.3 Aetiology/risk factors

More than 90% of ovarian cancer patients have no family history of the disease, suggesting no major familial determinant in the majority of cases. Therefore, most cases are likely to arise as a result of spontaneous or environmentally induced carcinogenesis. Some risk factors associated with the disease are presented here.

i) Environmental factors

A strong geographical variation of the disease has been observed. In general industrialised or "Westernised" countries have a higher incidence than other countries (Parazzini et al., 1991). Interestingly, immigrants from a country of low incidence (Japan) to a country of high incidence (USA) and especially second generation immigrants show levels of incidence comparable to those found in their new country (USA) (Herrinton et al., 1994).

ii) Hereditary factors

The ovarian cancer cases which are thought to be caused by clearly defined hereditary factors account only for a small percentage of the cases. Women with one first degree relative with ovarian cancer have a 5% increase in risk while women with 2 first degree relatives suffering from the disease have a 7% increase in risk . However, women with defined genetic syndromes run a 40-50% level of risk for developing the disease (Narod., 1994). These syndromes, can be (a) *hereditary syndromes,* where patients appear with bilateral and multifocal tumours (b) *hereditary breast/ovarian cancer,* where patients develop breast and ovarian cancer and in which tumour suppressor genes such as BRCA1 seem to be involved in these cancers, (c) *hereditary site-specific ovarian cancer,* and (d) *Lynch syndrome II* , which are characterised by the association of hereditary colorectal cancer with endometrial, ovarian, gastrointestinal or breast cancer (Lynch et al., 1985, 1993).

iii) Reproductive factors

About 10-20% of ovarian cancer patients respond to anti-oestrogens and about 36% to progestins. In post-menopausal women, PR positivity is

associated with well differentiated ovarian tumours while most endometroid tumours contain relatively high amounts of PR. PR content in tumours has been linked with prognosis but there is not complete agreement on these results. ER levels have also been investigated in tumours and the evidence that they might be linked with prognosis of the disease is conflicting. The aetiology of ovarian cancer can be related to the endocrine hormonal system. Fathala (1971) proposed the "Incessant ovulation" theory, according to which repeated ovulatory cycles drive the ovarian cancer surface epithelium to repeated proliferative repair cycles which might cause genetic damage and lead to ovarian cancer. Factors which reduce the number of ovulatory cycles also seem to decrease the levels of risk. For example, nulliparous women have an increased risk of ovarian cancer compared to women with children. Lactation seems to decrease the risk even further. Women with early menarche or late menopause have an increased risk of ovarian cancer. Women who undergo fertility treatment with gonadotrophins and stimulants of the pituitary gonadotrophins, also have an increased risk for developing ovarian and endometrial cancer. On the other hand the use of oral contraceptives seems to decrease the rate of risk of the disease by 30-60% and the effect seems to last for a number of years after the use of the drug has been stopped (Ron et al., 1987; Whittemore et al., 1992, 1993; Spirtas et al., 1993; Rossing et al., 1994).

iv) Dietary factors

One of the strongest risk factors concerning diet is the consumption of saturated fat. Every 10 g of saturated fat consumption increases the risk of ovarian cancer by 20% while consumption of the same amount of vegetable fibre reduces the risk by 37% (Risch et al., 1994). Although no relation has been demonstrated between oestrogen ingestion and increased ovarian cancer risk a considerable amount of oestrogen and related compounds are present in saturated animal fat as well as a number of different products associated with "Westernised" societies in the form of preservatives, and other ingredients (Risch et al., 1994;

Adlercreutz et al., 1994). Other dietary factors such as consumption of coffee, alcohol and fish have been associated with increased risk but experimental data have not proven strong (Whittemore et al., 1988; Byers et al., 1983).

1.2.4 Prognosis

Extent of disease spread at the time of diagnosis is the most powerful prognostic factor in ovarian cancer (Griffiths et al., 1975). Because of the extremely health threatening nature of the disease when diagnosed at a late stage, there is an increased need for better prognostic factors. One example of a biological factor, which has already been correlated with survival, is the level of CA-125 protein in serum (Sevelda et al., 1989). CA-125 is a glycoprotein expressed by a number of different epithelia, including mullerian derived epithelia. CA-125 is expressed at higher levels in almost 80% of ovarian cancers. The fall of CA-125 levels after therapy, correlates well with the response of the patient to treatment. Other possible biological and genetic factors could eventually be related with better prognosis.

1.2.5 Treatment

i) Surgery

In order to establish diagnosis and evaluate the stage of ovarian cancer accurately, surgery must be undertaken. Surgery is the standard treatment for stage I disease with removal of the tumour, the uterus (hysterectomy), the fallopian tubes and contralateral ovary (bilateral salpingo-oophorectomy). In about 50% of cases in more advanced stages, surgery is necessary for the patients comfort, and wherever possible, cytoreduction of the tumour is performed. This is because (i) maximum possible tumour size reduction increases chances of responding to adjuvant therapy and (ii) during the progression of the disease there is increasing tumour growth in the peritoneal cavity which can lead to the development of intestinal obstructions. In such cases surgical removal of

these obstructions can provide comfort for the patients. Most accurate assessment of the response to any therapy, is by repeated laparotomy.

ii) Radiotherapy

Radiotherapy remains a controversial method of treatment. Because of the complicated spread of the disease, effective radiotherapy must cover the entire abdominal cavity. However, such practice is limited because of the sensitivity of the surrounding organs (liver, kidneys) to radiation. Radiotherapy is used mainly for cases with poor prognostic features and in patients at a more advanced stage of the disease.

iii) Chemotherapy

Cytotoxic chemotherapy is now commonly used in patients with advanced disease as first line adjuvant therapy. Combination chemotherapy improves the 5 year survival of this disease by 20% (Omura et al., 1986). Most of the standard chemotherapeutic treatments consist of platinum containing compounds with alkylating agents. A number of possible new chemotherapeutic agents have been discovered and are already in clinical trials. Some of these new agents are members of the family of *Taxans*. Taxans are natural and semi-synthetic diterpenoids based on the structure of the natural product*Taxol* which was first isolated from the western yew tree in 1971. The use of such compounds alone or in combination with cisplatin have shown promising results in clinical trials (Einzig et al., 1992; Thigpen et al., 1994).

1.2.6 Growth regulation of ovarian cancer

i) Hormones

Oestrogen can stimulate growth of ovarian cancer cell lines which express oestrogen receptor (ER) at levels between 20 and 30 fmol/mg of protein (Langdon et al., 1990; Chien et al., 1994) while such an effect is not seen in cell lines expressing lower ER levels (Langdon et al., 1990, 1994). It has also been shown that oestrogens induce a number of other factors which are thought to play a role in growth of normal and tumour cells. In ER positive ovarian cancers 17ß-oestradiol (E_2) has been shown to

downregulate ER and EGF receptor content and to increase the levels of TGF-α mRNA and the secretion of procathepsin D (Nash et al., 1989; Galtier-Dereure et al., 1992; Crew et al., 1992; Rowlands et al., 1993). Addition of anti-oestrogens to ER positive cell lines can block the effect of E_2 on the growth of these cells and tamoxifen can be effective in ER positive ovarian primary tumours (Lazo et al., 1984; Nash et al., 1989; Langdon et al., 1990, 1992).

ii) Growth factors

A number of studies using ovarian cancer cell lines and xenografts, have suggested possible involvement of several growth factors in the development of ovarian cancer. Epidermal growth factor (EGF) has been most extensively studied. This growth factor demonstrates considerable similarity with transforming growth factor-α, (TGF-α) amphiregulin and cripto. Studies in ovarian tumours and ovarian cancer cell lines have demonstrated the presence of EGF and TGF-α (30 to 70% and 50 to 100% of cases respectively) (Morishige et al., 1991; Kohler et al., 1992; Stromberg et al., 1994). Exogenous addition of these two growth factors in ovarian cancer cell lines *in vitro* demonstrated their ability to exert mitogenic effects (Scambia et al ., 1991; Zhou and Lenng 1992; Crew et al., 1994) through binding and activation of the same receptor, the EGF receptor (EGFR). EGFR is a 170 kD glycosylated transmembrane protein, present in 33 to 70% of ovarian cancers (Morishige et al., 1991; Henzen-Logmans et al., 1992). Investigations in ovarian carcinoma cell lines have confirmed the presence of EGFR and secretion of EGF and TGF-α suggesting a possible autocrine control of growth (Kurachi et al., 1991; Jindal et al., 1994). EGF receptor levels may relate to poor prognosis in ovarian cancer (Bataglia et al., 1989; Owens et al., 1991). The EGF receptor (also known as c-erbB-1) has similarities with the c-erbB-2, c-erbB-3 and c-erbB-4 receptors. C-erbB-2 and c-erbB-3 proteins are overexpressed in ovarian tumours (20 - 30% and 89% respectively) and their overexpression associated with poor survival (c-erbB-2) or with borderline and early invasive lesions (c-erbB-

3) (Slamon et al., 1989; Berchuck et al., 1990; Simpson et al., 1995). TGF-ß peptides (TGF-ß1, TGF-ß2 and TGF-ß3) exert growth inhibitory effects in normal ovarian epithelial cultures, ovarian cancer cultures and almost 50% of ovarian carcinoma cell lines (Berchuck et al., 1990, 1992; Marth et al., 1990; Bartlett et al., 1992;). The proposed role for TGF-ß peptides is that of controlling the growth of normal ovarian epithelium and such control might be lost at an early stage of malignant transformation in the ovary (Havrilesky et al., 1995). Insulin-like growth factors I and II (IGF-I, IGF-II) are, as the name implies, structurally related to insulin. Both IGFs and their receptors (insulin receptor, IGF receptors) have been identified in ovarian cancer cell lines (Yee et al., 1991; Bartlett et al., 1995) and ovarian tumours (Foekens et al., 1990; Beck et al., 1994) Exogenous addition of IGFs to the same cell lines may stimulate growth suggesting a possible autocrine control of growth (Resnicoff et al., 1993). Other growth factors such as fibroblast growth factor (FGF) and platelet derived growth factor (PDGF) and their receptors are also expressed in ovarian tumours and are associated with effects on the surrounding stromal components (Sariban et al., 1988; Versnel et al., 1994; Di Blassio et al., 1993; Crickard et al., 1994).

iii) Cytokines

Cytokines families include the interleukins, interferons and tumour necrosis factor (TNF). Cytokines are believed to regulate the growth of many tumours, as well as being able to regulate oestrogen and growth factor effects.

A number of cytokines are thought to be involved in ovarian cancer pathogenesis. Interleukins 1α (IL-1α) and 1ß (IL-1ß) are both produced by ovarian cancer cell lines *in vitro*. IL-1α has been shown to stimulate proliferation of ovarian cancer cells in culture and it is thought to stimulate the expression of tumour necrosis factor (TNF) (Wu et al., 1992). Interleukin 6 (IL-6) concentrations are elevated in women with ovarian cancer (Watson et al., 1990; Berek et al., 1991) and experiments in which the autocrine IL-6 control system was blocked in ovarian cancer cell lines, resulted in decreased growth of the ovarian cancer cells

(Watson et al., 1993). Other cytokines are also thought to be involved in the inhibition of growth of ovarian cancer cells (IL-4) and in the inhibition of the cytokines involved in the system (IL-10) (Berek et al., 1994). TNF is expressed in 70% of ovarian tumours (Naylor et al., 1993) and addition of recombinant TNF-α to ovarian cancer cell lines has been shown to stimulate growth and also expression of TNF-α mRNA (Wu et al., 1992). Interferons (IFN) are members of another family of cytokines that are thought to be cytotoxic and to inhibit the growth of ovarian cancer cells (Malik et al., 1991). α-IFN, ß-IFN and γ-IFN are already used in clinical trials and these have shown good activity against minimal residual disease (Willemse et al., 1990; Cappelli et al., 1992; Colombo et al., 1992; Markman et al., 1993).

1.2.7 Oncogenes and tumour suppressor genes in ovarian cancer

i) Oncogenes

Mutations in normal cellular genes which control cell growth and proliferation can produce malignant transformation. Such mutations may convert normal proto-oncogenes into *oncogenes*. A number of oncogenes have been associated with ovarian cancer. The c-myc proto-oncogene is responsible for the expression of a protein which controls the proliferation, differentiation and programmed cell death (apoptosis) of the cell and seems to be overexpressed mainly in serous ovarian cancers (Yokota et al., 1986; Kikuchi et al., 1994).

Studies have revealed that 20% of ovarian cancers show amplification of the erbB-2 gene and overexpression of the encoded protein (Slamon et al., 1989; Berchuck et al., 1990a). Overexpression of K-ras gene is usually associated with advanced ovarian cancers (Chien and Chiau, 1993; Park et al., 1995). The extensive study of such oncogenes and their role in normal and malignant cells could potentially lead to valuable information regarding the prognosis and/or development of cancer.

ii)Tumour suppressor genes

Tumour suppressor genes are defined as genes involved in the control of

normal cell growth and whose loss or inactivation is associated with the development of malignancy. Tumour suppressor genes include the retinoblastoma (RB-1) gene which is involved in the regulation of normal cell growth by restricting cell cycle progression into the G_1 phase of the cell cycle (Borg et al., 1992; Bowcock et al., 1990) and the p53 gene which encodes for p53 protein and is overexpressed as a result of DNA damage driving the cells to cell cycle arrest at the G_1-S phase or into apoptosis (Levine et al., 1992; Prives et al., 1993).

A recent study by Zhang et al., (1995) has suggested that p53 mutations may be found in almost 50% of ovarian cancer cases studied whereas no mutations in the same gene have been detected in benign tumours. In the benign regions surrounding the ovarian tumours containing the alteration of the p53 gene, the same mutation was observed. In many ovarian tumours the wild type BRCA1 allele appears to be lost either due to a germ-like mutation or (most commonly in ovarian cancer) through loss of heterozygosity on 17q chromosome (Eccles et al., 1990; Cornelis et al., 1995; Takahashi et al., 1995). There is a high risk of development of ovarian cancer in women with BRCA1 gene mutations, this ranging between 20 and 80% (Easton et al., 1993).

1.3 Breast cancer

1.3.1 Introduction/epidemiology

Breast cancer is the second most common female malignancy, after lung cancer, in the majority of developed countries and the most common female malignancy in the UK. It is also the predominant cause of death for women between the age of 35 and 55 in Western societies. Each year about 150,000 women are treated for the disease in the UK with 30,000 new cases and over 15,000 deaths, while in the USA it has been estimated that one woman dies from breast cancer every 13 minutes (Washington Post, 1990). According to a 1975 estimation, more than 500,000 breast cancer cases are diagnosed worldwide each year, and clearly still represents one of the greatest dangers for the health of women (Boyle,

1988).

1.3.2 Origins of breast cancer

The normal human mammary gland is a secretory organ, which consists of a number of ductules, separated by fibrous bands of connective and adipose tissues. The nipple comprises the ending of a branching system of lactiferous ducts which are formed by the coalescing system of terminal duct lobular units (TDLU), created by the interfusion of smaller groups of terminal ductules. The surface of all the duct systems, with the exception of the lactiferous ducts, is lined by a single epithelial cell layer, and it is from this layer that most breast cancers develop. During tumourigenesis, it is thought that epithelial cells proliferate without control, resulting in a multi-layered epithelium (hyperplasia), and as the disease progresses, the epithelium becomes even more abnormal (atypical hyperplasia) and eventually proliferates to fill the spaces. Because at this stage the lesion is restricted to the lobule and tumour cells have not penetrated the basement membrane it is referred to as "*in situ* cancer". *Ductal carcinoma in situ* (DCIS) consists of solid malignant cells in the lumen or cribriform (cells growing into the duct lumen) and is detectable by mammography. DCIS represents the last step before breast cancer becomes invasive. The stages of the progression of the disease are summarized in table 1.2 and involve the breaching of the basement membrane.

T-Tumour		
T1	Tumour 2 cm or less in greatest dimension	
	T1a	0.5 cm or less in greater dimension
	T1b	More than 0.5 cm but not more than 1 cm in greatest dimension
	T1c	More than 1 cm but not more than 2 cm in greatest dimension
T2	Tumour more than 2 cm but not more than 5 cm in greatest dimension	
T3	Tumour more than 5 cm in greatest dimension	
T4	Tumour of any size with direct extension to chest wall or skin	

N- Regional lymph nodes	
NX	Regional lymph nodes can not be assessed (e.g. Previously removed)
NO	No regional lymph nodes metastasis
N1	Metastasis to moveable ipsilateral axillary node(s)
N2	Metastasis to ipsilateral axillary node(s) fixed to one another or to other structures
N3	Metastasis to ipsilateral internal mammary lymph node(s)

M- Distant metastasis	
MX	Presence of distant metastasis cannot be assessed
MO	No distant metastasis
M1	Distant metastasis (includes metastasis to supraclavicular lymph nodes)

Stage grouping			
Stage I I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1,N2	M0
Stage IIIB	T4	Any N	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 1.2: TNM (tumour, node metastasis classification). Abbreviation from Hermanek P, Sodin LH (Eds). In: *TNM Classification of Malignant Tumors. UICC International Union Against Cancer.* Springer-Verlag Ed. (4th Ed.), Berlin, 1987; 93-99.

1.3.3 Aetiology/risk factors

A number of factors have been associated with an increased risk of developing breast cancer disease. These may be summarised in the following categories:

i) Gender

Breast cancer can affect both men and women. However, the incidence of breast cancer in women is a hundred times greater than in men suggesting that gender is an important risk factor.

ii) Environmental factors

The incidence of breast cancer in different geographical regions seems to vary substantially (Waterhouse 1982; Muir et al., 1987). More industrialised or "Westernised" countries seem to have significantly higher incidence (incidence in the USA is 5-fold higher than in Japan) (Henderson et al., 1993). Populations which have migrated from countries with low risk to countries with higher risk show increased incidence with successive generations (Stemmerman et al., 1970; Locke and King 1980). Substantial regional variations may also be seen within individual countries.

iii) Ionizing radiation

Ionizing radiation is the only definite cause of breast cancer. Studies have shown that exposure to high levels of radiation (Nagasaki, Hiroshima) resulted in increased development of the disease (MacGregor et al., 1977).

iv) Hereditary factors

Only 7% of the total breast cancer patient population show true hereditary aetiology which has been determined by medical genetic scrutiny. Increased risk of the disease seems to be associated with women who had a first degree relative with breast cancer (1.5 to 2-fold risk), but there is a 5 to 6-fold risk for those with two affected relatives. Premenopausal women with an affected first degree relative have a 3 to 5-fold increased risk or a 6 to 7-fold risk if that relative was identified with bilateral cancer.

v) Benign breast disease

There is controversy as to whether women who have been diagnosed

with benign breast disease have an increased risk of developing breast cancer and that risk seems to be quite variable but generally modest. However, women with ductal or lobular hyperplasia may have a 5-fold increased risk, which could be doubled if they also have a family history of the disease.

vi) Reproductive life/childbearing

Multiple studies have suggested that an extended reproductive life increases the risk of developing breast cancer (MacMahon et al., 1973). These, in association with the observation that the menopause decreases risk of developing breast cancer, suggest that steroid hormones may play an important role in breast cancer. Thus women with late menarche and menopause before the age of 45 show a decreased risk of developing breast cancer in comparison to women with early menarche and late menopause (Trichopoulos et al., 1972). A possible explanation for such observations could be the extended exposure of women with prolonged reproductive life, to ovarian hormones such as oestrogen and progesterone which can effect the proliferation rate of hormone sensitive breast cells.

Childbearing, which disrupts the menstrual cycle, seems to protect against the overexposure of women to such hormones. Prolonged lactation together with a high number of pregnancies reduce the risk. It has been shown that nulliparity increases the risk of breast cancer by 1.4-fold, women who had their first pregnancy within a few years of menarche, compared to women who had their first pregnancy after 30 years of age seem to have a 50% decreased risk for the development of the disease.

vii) Body weight

In post-menopausal women there is a positive association between body weight and risk and an even higher association with mortality, perhaps because obese women are usually diagnosed at a later stage (de Waard and Baander-van Halewijn 1974).

viii) Other factors

The exogenous administration of oestrogens to women during

pregnancy, after the menopause or as a form of contraceptive may influence the risk of the disease. The use of oestrogens and progestogens as oral contraceptives has not appeared to increase the incidence of breast cancer in general however there is some concern about the use of oral contraceptives before first pregnancy, or before the age of 25 (Pike et al., 1981; MacPherson et al., 1987).

1.3.4 Prognosis

The prognosis of breast cancer varies quite dramatically among individuals and very much depends on the stage at which the disease was diagnosed (Table 1.2). Some patients live for more than 20 years and are then cured while others die very shortly after they had been diagnosed and despite heavy treatment. In general, patients with metastatic disease have a low survival rate in comparison with patients with tumours restricted to the breast. Because of the severity of the disease and the great variability between patients, the need for better prognostic methods is obvious (Tobar et al., 1992).

1.3.5 Treatment

i) Surgery

Surgery is still the main treatment for invasive breast cancer which is confined to the breast. In the last years, the general practice is to reduce the extent of surgery only to local excision of the tumour rather than radical mastectomy.

ii) Radiotherapy

In breast cancer, radiotherapy has been used for localised lesions as it has not been effective and cannot be used for widespread disease due to its potentially harmful side-effects. For a number of years, radiotherapy has been used in combination with local surgery to eradicate malignant cells in residual breast tissue.

iii) Chemotherapy

Breast cancer, is moderately sensitive to chemotherapy. Adjuvant

systemic therapy, is routinely offered to women with early signs of the disease and especially to those with invaded axillary lymph nodes. However, the relapse of the disease in these patients is associated with lowering of the responsiveness to systemic treatment. The basic endocrine treatment in breast cancer consists of drugs that reduce the oestrogenic effects on growth. Tamoxifen has been the major drug used to block the binding of oestrogen to its receptor (Rubens et al., 1988) and has also been shown to induce the production of TGF-ß which may play an inhibitory role in the growth of tumours (Butta et al., 1992). However, resistance to tamoxifen has been shown in many tumours possibly as a result of mutations to oestrogen receptors (Jiang and Yordan 1992; Fuqua et al., 1993). A number of chemotherapeutic drugs have also been used against breast cancer including doxorubicin, methotrexate, mitomycin C and adriamycin. Although adriamycin has been the most effective single agent (Henderson et al., 1989), it has been suggested that several drugs need to be used in the treatment of breast tumours and to cope with drug resistance (Gregory et al., 1993). In cases where patients are at very advanced stages, systemic therapy is used to relieve symptoms.

1.3.6 Growth regulation of breast cancer

The growth of breast cancer cells appears to be under the regulation of a variety of hormones, growth factors and cytokines. Oncogenes and tumour suppressor genes may produce dys-regulated growth and allow malignant transformation. These effects are described below.

i) Hormones

Studies on the effects of steroids on breast cancer cells growing *in vitro* have shown that these hormones are involved in the growth control of the malignant cells in the breast. ER-positive breast cancer cell lines such as MCF-7 can be stimulated by 17ß-oestradiol while ER negative cell lines such as MDA-MB-231 are not (Clark et al., 1989). In hormone-sensitive breast cancer cell lines, it has also been suggested that steroid hormones induce proteins such as cathepsin D and TGF-α which may be important

regarding tumour spread (Lippman and Dickson 1989). Steroid hormones appear to have no effects on the growth of ER-negative (or low ER) cell lines (Engle et al., 1978).

ii) Growth factors

The development of the normal breast may be influenced by growth factors (Lippman and Dickson 1989) such as epidermal growth factor (EGF) and TGF-α which increase proliferation of normal breast cells in culture (Perushinge et al., 1992); TGF-α can also be detected in proliferating human mammary epithelium. Anti-EGF receptor antibodies block the effects of TGF-α suggesting that it acts through the EGF receptor (Bates et al., 1990). The EGF receptor and a related protein (p185) which is encoded by the c-erbB2 oncogene are over-expressed in a minority (20-30%) of breast cancers (Klijn et al., 1992; Fox et al., 1994). IGF-I receptor expression is also over-expressed in a number of breast cancers (Pekonen et al., 1988) and may therefore be associated with malignant transformation. The TGF-ß family consists of 3 isoforms which are able to inhibit growth of epithelial cells (Knabbe and Zugmaier 1994). However, recent studies have associated TGF-ß expression with rate of the disease progression and modulation of angiogenesis as well as neovascularization and progression of tumours towards hormone independence (Dickson et al., 1986). In general, breast cancer cells appear to produce and secrete higher amounts of growth factors and over-express receptors for such factors. Such effects may result in unregulated proliferation of tumour cells (autocrine action) but may also induce mitogenesis and other effects on surrounding cells (paracrine action).

iii) Cytokines

Some cytokines are differentially expressed in tumours compared to normal breast tissue. IL-6 expression is high in normal tissue but low in almost all tumours while TNFα which is absent in normal breast epithelium is widely expressed in breast cancers (Basolo et al., 193; Pusztai et al., 1994).

1.3.7 Oncogenes and tumour suppressor genes in breast cancer

i) Oncogenes

In human breast carcinomas, alterations in c-myc were found in 25% of tumours (Escot et al., 1986; Berns et al., 1992) and have been associated with increased aggressiveness and poor prognosis. Mutations in the oncogenes H-ras and K-ras, have been identified in breast cancer cell lines (Bradbury et al., 1991; Kraus et al., 1984) but only rarely found in breast carcinomas. Amplification of the c-erbB-2 gene is found in 20-30% of breast cancers and has been shown to correlate with aggressive features in invasive tumours (Slamon et al., 1987). Another proto-oncogene, CCND1 encodes for cyclin D, a protein regulating progression in the G_1 phase of the cell cycle. Amplification of this gene leads to overexpression of cyclin D-1 seen in many breast cancers (Bartkora et al., 1994; Gillet et al., 1994).

ii) Tumour suppressor genes

Alterations in RB-1 have been found in breast carcinomas and breast cancer cell lines while mutations in the p53 gene have been reported in a number of cancers and in about 60% of breast cancers (Mackay et al., 1988; Varley et al., 1991) and have been associated with aggressive tumours. Other studies have suggested that mutations in the genes responsible for genetic stability, DNA replication, or DNA repair found in many breast cancer can account for damage of the genome (Loeb, 1994). BRCA-1 and BRCA-2 are tumour suppressor genes (Friedman et al., 1994) linked with the onset of inherited breast cancer which accounts for 5% of the disease.

1.4 Endothelins

1.4.1 Discovery

In the 1980s, it was shown for the first time that the endothelium was involved in the contraction of smooth muscle. The discovery of a peptidergic endothelium-derived contracting factor (EDCF) with, until that point, unknown properties was made when cultured medium from bovine endothelial aortic cells was added to porcine smooth muscle cells from isolated pig coronary arteries (Hickey et al., 1985). The experimental data suggested a slowly developing, long-lasting contraction of the smooth muscle, an observation which was later confirmed by a number of other groups (Gillesbie et al., 1986; O'Brien et al., 1987). The EDCF peptide was later isolated, purified, cloned and named *endothelin-1* (Yanagisawa et al., 1988 a,b) and was characterised as the most potent vasoconstrictor known. Since then, a number of studies have more clearly defined the biological role of the endothelin peptide family, and provided a better understanding of its involvement in the pathomechanisms of disease.

1.4.2 Structures of the endothelin peptides

Recently, three different ET genes have been identified in distinct chromosomal locations expressing 3 different endothelin isopeptides (ET-1, ET-2, ET-3) in animals and humans (figure 1.1). The endothelin isoforms are initially synthesised as larger preproendothelins which are cleaved to 37-41 amino acid peptides ("big" endothelins) by specific endopeptidases (figure 1.2). The mature endothelins are products of the proteolytic cleavage of big endothelins. However, such cleavage is not performed in all big endothelin propeptides since they have been identified in plasma (Miyauchi et al., 1989) and in the conditioned media of cultured cells (Parker et al., 1992). All endothelins show marked homologies to a family of peptides known as Sarafotoxins (SRTXs) found

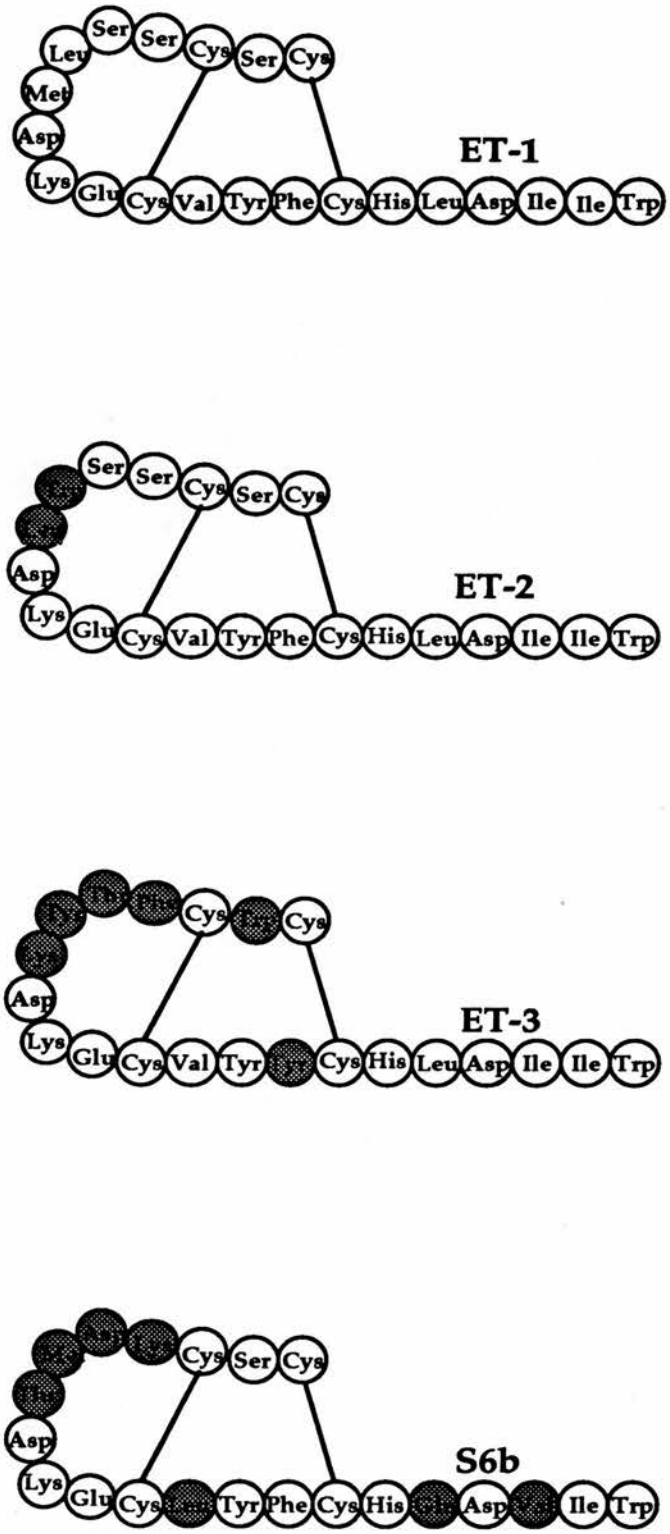


Figure 1.1: The primary sequences of endothelins (ET)-1, -2 and -3 showing the positions of the disulphide bonds and residues that differ from those of endothelin-1 (shaded). The snake venom, sarafotoxin S6B is shown for comparison (original figure taken from Huggins et al., 1993).

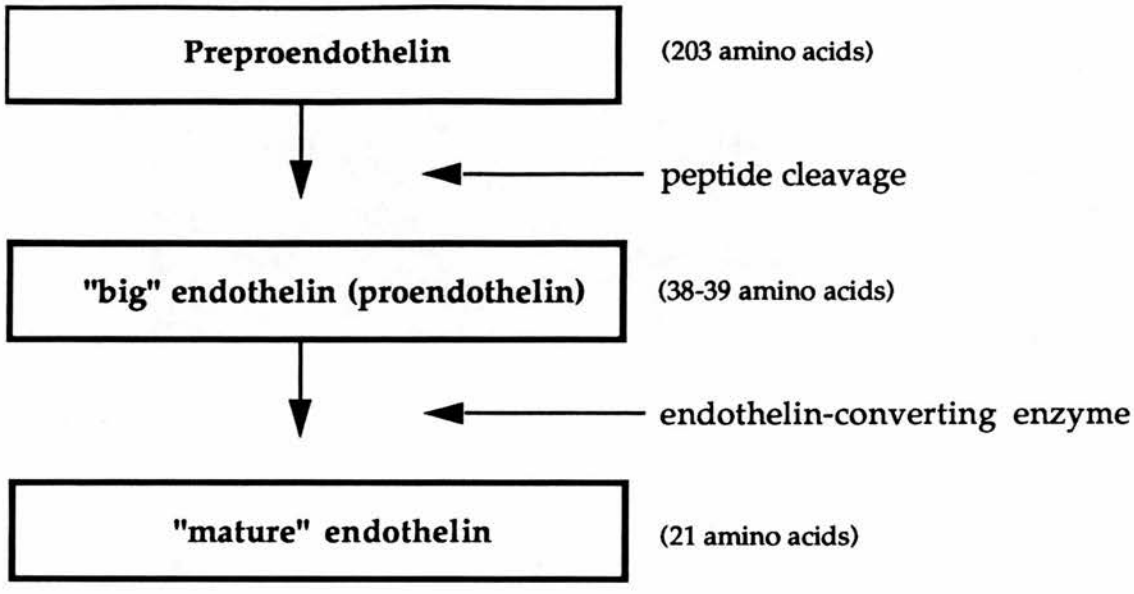


Figure 1.2: The biosynthesis and processing of endothelin from preproendothelin (original illustration in Gandhi et al., 1994).

in the venom of a rare snake, *Atractaspis-engaddensis*. Four SRTXs have been identified and together with the three endothelin peptides form the endothelin family. All the family members consist of 21 amino acid residues and are identical at 10 amino acid positions. The COOH region of the peptides is highly hydrophobic and shows extensive homology between different members of the family. The amino acids that differ between members of the family are, with very few exceptions, still similar in terms of charge and hydrophobicity. The four Cys residues in the sequence of all endothelins have important structural roles, forming two disulphide bonds (S-S bonds between Cys^{1}-Cys^{15} and Cys^{3}- Cys^{11}) which fold the peptide and are vital for the affinity of binding with at least one type of endothelin receptors (ET_A-R). The S-S bonds found in endothelins are different from the common S-S bonds present in other biological molecules. Studies on the physical properties of the ET peptides using proton NMR measurements and distance geometry calculations (Dalgarno et al., 1992; Endo et al., 1989; Munro et al., 1991; Reily and Dunbar 1991) have suggested that ETs have a very compact formation when in solution, exhibiting little solubility in aqueous solutions at neutral pH as well as low partition into ether/water interfaces (Spinella et al., 1989) and form micelles at 22 μM concentrations (Bennes et al., 1990). Most of the NMR studies have been performed in the presence of organic solvents or at non-physiological pH ranges and in aqueous/organic solvent mixtures. Although there have been different suggestions about the tertiary structure of the peptides, such studies have come to some common conclusions. The central part of the peptide forms a helix structure which is not of the regular α-helix type. There is some controversy as to whether the helical region is between residues Leu^{6} and Cys^{11} with both S-S bonds incorporated into the structure (Endo et al., 1989; Saudek et al., 1991) or between Leu^{6} and Cys^{15} (Munro et al., 1991) thus including only one S-S bond. According to an extended study on the N-terminus of the endothelin peptides, it has been proposed that it constitutes the area including the key structural region for the

differentiation of the binding activity at the ET_A-R and ET_B-R receptor sites. No common conclusions have been drawn regarding the COOH terminal region of the ET peptides. Two models with distinct differences have been suggested. The first, suggests that the tail folds back towards the helical region (Saudek et al., 1991) and the second that the COOH tail is flexible and can form a number of different conformations (Endo et al., 1989; Munro et al., 1991). During the last two years a study by Janes et al., (1994) determined the crystal structure of human ET-1 at 2.18Å resolution in a purely aqueous medium. According to this study, the peptide consists of an N-terminal ß-strand which is extended, followed by a loop of hydrogen bonds involving the carbonyl oxygen of residue 7 and the amide proton of residue 11. According to the same study, the region following the loop of hydrogen bonds forms an ordered but irregular helix which is stabilised by main chain to main chain and main chain to side chain hydrogen interactions and the two S-S bonds between Cys^1-Cys^{15} and Cys^3-Cys^{11}. That helix may represent a recognition site for endothelin converting enzyme (ECE) resulting in the binding of the converting enzyme and the cleavage of "big" ET to the mature ET polypeptide. According to the final NMR structure, the COOH terminus of the molecule is helical in its crystal structure. The question however remains as to whether the X-ray crystallographic conformation represents the natural biological form of the molecule or whether the peptide is different in solution. The structure of the COOH terminal region, responsible for the binding and biological activity remains to be clarified.

1.4.3 Endothelin genes

Three distinct ET related genes in humans and other mammalian species have been identified using ET-1 gene fragments in order to screen genomic libraries (Itoh et al., 1988; Kimura et al., 1989a; Inoue et al., 1989 a,b; Onda et al., 1991). Human ET-1, ET-2 and ET-3 genes have been mapped on chromosomes 6 (Arimori et al., 1991a; Bloch et al., 1989a,b), 1(Arimari et al., 1991; Bloch et al., 1989a; Hoehe et al., 1993) and 20

(Arimari et al., 1991; Bloch et al., 1989a; Rao et al., 1991) respectively. Considering the structure of the human ET genes, more information is known regarding the ET-1 gene than the ET-2 and ET-3 genes. The 6.8 Kb coding region of the ET-1 gene consists of five exons which are interrupted by four introns. The primary transcript mRNA is 2.3 Kb in length and directs the translation of the prepro-ET-1 peptide. Each exon encodes a portion of the prepro-ET-1 which includes the sequences of "big" ET-1 and ET-1 peptide. The ET-1 promoter was located at 9 bp downstream of a CAAT box and includes a number of regulating sequences [TATAA box, binding sites for nuclear factor-1 (NF-1), GATA-2, AP-1/jun and acute phase reaction regulatory elements (APRE) (Benatti et al., 1993; Inoue et al., 1989a,b; Gronostajski 1987)].

1.4.4 Endothelin expression

Since the original isolation of ET-1 from porcine aortic endothelial cells (Yanagisawa et al., 1988b), ET isoforms have been detected in a number of tissues. Most studies have used immunological methods which can detect ET and "big" ET isoforms in the femtomole range but cannot fully characterize the identity of the ET isoform because of cross-reactivity of the antibody with the different ET isoforms. Qualitative data regarding gene expression has also been obtained with the use of Northern analysis, RT-PCR and in situ hybridisation techniques (Baley et al., 1990; Economos et al., 1992, O'Reilly et al., 1992; Pagotto et al., 1995; Pekonen et al., 1995).

Of the isoforms, ET-1 is the major form produced by all endothelial cell types such as the umbilical vein (Inoue et al., 1989a,b), glomerulus (Marsdon et al., 1991), aorta (Tokunaga et al., 1992), mesenteric artery (Dohi et al., 1992), brain microvessel (Yoshimoto et al.,1990) and corpus covernosum (Saenz de Tejada et al., 1991). Vascular smooth muscle cells also express ET-1 mRNA and protein (Resing et al., 1990). ET-1 is also present in a number of other cell types such as breast epithelium (Baley et al., 1990), endometrial stromal glandular epithelial cells (Economos et al., 1992a,b), keratinocytes (Imokawa et al., 1992), bone marrow mast cells and

macrophages (Ehrenreich et al., 1992) and cardiomyocytes (Suzuki et al., 1990). Equally, a number of tumour derived cells also express ET-1. These include, endometrial carcinoma (Economos et al., 1992a,b), Hep-2 hepatocarcinoma and HeLa cells (Shichiri et al., 1991a,b), renal adenocarcinoma (Tokito et al., 1991), MDA-MB-231, MCF-7, ZR-75-1 human epithelial breast cancer cells (Yamashita et al., 1993), mammary carcinomas (Kusuhara et al., 1990; Patel et al., 1995), pancreatic tumour samples (Nelson et al., 1995), SKOV-3, OVCA433, A2780 and BG-1 ovarian carcinoma cells (Bagnato et al., 1995). In contrast ET-2 and ET-3 have not been detected in either vascular endothelium or smooth muscle as suggested by Northern analysis (Bloch et al., 1989a,b; Firth, 1992) but both are expressed in human endometrium (O'Reilly et al., 1992). ET-2 has also been shown to be expressed in human renal adenocarcinoma (Tokito et al., 1991). At the tissue level, mRNA for ET-1 has been shown to be present in all organs examined with lung being the predominant site expressing ET-1 mRNA 5-fold higher than the large intestine and 15 times higher than other organs (Onda et al., 1990; Firth and Ratchlife 1992). ET-3 mRNA is also detected at higher levels in a number of organs. Table 1.3 summarises the cell lines and tissues where ET peptides have been shown to be expressed. Studies on the regulation of ET-1 expression indicate that in endothelial cells, levels of ET-1 mRNA were increased after treatment with growth factors, cytokines and vasoactive substances such as thrombin (Kurihara et al., 1989; Emori et al., 1992), TGFß (Kurihara et al., 1989; Yanagisawa and Masaki 1989), TNF-α

Normal tissue/cell type	Reference
breast epithelium	Baley et al., 1990
cardiomyocytes	Suzuki et al., 1990
endometrial stromal cells	Economos et al., 1992a
glandular epithelial cells	Economos et al., 1992b
bone marrow	Ehrenreich et al., 1992
mast cells	Ehrenreich et al., 1992
macrophages	Ehrenreich et al., 1992
keratinocytes	Imokawa et al., 1992
Tumour-derived cells	**Reference**
pulmonary squamous cell carcinoma	Giaid et al., 1990
pancreatic tumour samples	Kusuhara et al., 1990, Nelson et al., 1995
mammary carcinomas	Kusuhara et al., 1990 Patel et al., 1995
Hep-2 hepatocarcinoma	Scihiri et al., 1991a,b
HeLa cervical carcinoma cells	Scihiri et al., 1991a,b
renal adenocarcinoma cells	Tokito et al., 1991
endometrial carcinoma	Economos et al., 1992a
human endometrial adenocarcinoma cells	Pekonen et al., 1992
MDA-MB-231, MCF-7, ZR-75-1 human breast epithelial cancer cells	Yamashita et al., 1993
SKOV-3, OVCA433, A2780, BG-1 human ovarian epithelial cancer cells	Bagnato et al., 1995

Table 1.3: List of the normal and malignant tissues and cell lines that have been shown to express and/or secrete endothelin peptides.

(Lamas et al., 1992), interleukin-1 (Maemura et al., 1992), angiotensin II (Dohi et al., 1992), bradykinin (Marsden at al., 1991) and insulin (*in vitro*) (Oliver et al., 1991; Metsärine et al., 1994). ET mRNA levels were increased in cells of the haemopoietic system (human macrophages) following the addition to cultures of lipopolysacharides and phorbol esters (Ehreneich et al., 1990); in human breast epithelial cells by supplementing culture media with prolactin and in human breast cancer cells by IL-6 and bombesin (Yamashita et al., 1993). In endometrial stromal cells and cultured human avascular amniotic cells, EGF, IL-1 and TNF-α all increased ET expression (Casey et al., 1991). Some of the factors that have been shown to modulate the production of ETs are shown in table 1.4. The concentrations of the mature peptides in circulating blood of healthy individuals and patients with a variety of diseases have been measured by radioimmunoassays specific for ET-1, ET-3 and big endothelin (Kurihara et al., 1989). Results from such studies suggested that the ET concentrations detected in samples from healthy individuals were lower than those in samples from patients.

System	Modulator	Effect	Reference
endothelial cells	thrombin	↑ ET-1 mRNA	Kurihara et al.,1989
endothelial cells	TGFß	↑ ET-1 mRNA	Kurihara et al.,1989
human breast epithelial cancer cells	prolactin	↑ ET-1 mRNA	Baley et al., 1990
haemopoietic system, (human macrophages)	lipopolysacharides phorbol esters	↑ ET-1 mRNA	Ehreneich et al., 1990
endometrial stromal cells and cultured human vascular amniotic cells	EGF IL-1 TNFα	↑ ET-1	Casey et al., 1991
endothelial cells	bradykinin	↑ ET-1 mRNA	Marsden et al ., 1991
endothelial cells	insulin (*in vitro*)	↑ ET-1 mRNA	Oliver et al., 1991 Metsarine et al., 1994
endothelial cells	angiotensin II	↑ ET-1 mRNA	Dohi et al., 1992
endothelial cells	INF TNFα	↑ ET-1 mRNA	Lamas et al., 1992 Maemura et al., 1992
endothelial cells	IL-1	↑ ET-1 mRNA	Maemura et al., 1992
human epithelial breast cancer cell lines	bombesin	↑ ET-1	Schrey et al., 1992
human epithelial breast cancer cell lines	IL-6	↑ ET-1	Yamashita et al., 1993

Table 1.4: List of molecules which have been shown to modulate the expression of endothelin-1 in different cell types.

1.4.5 Endothelin Converting Enzyme (ECE)

A number of enzymes have been proposed as endothelin converting enzymes including chymotrypsin, several aspartic proteases (eg. pepsin, Takaoka et al., 1990) and cathepsins D and E (Lees et al., 1990). The conversion of "big" endothelins to active endothelin peptides requires the cleavage of specific amino acid bonds. All the above mentioned enzymes, successfully cleave either one but not all bonds or they cleave more than the necessary bonds. Studies using macromolar concentrations of Pr [(N-a-rhamnopyranosyl-oxyhydroxyphosphinyl)-Leu-Trp], an inhibitor of the bacterial zinc protease, thermolysin, and zinc peptidase E-24.11 demonstrated that they could inhibit exogenous "big" ET concentrations both *in vivo* and *in vitro*. ECE may be structurally and catalytically similar to E-24.11 (Turner et al., 1993). Although ECE can be separated from E-24.11 by immunoaffinity chromatography or by isoelectric focusing (Murphy et al., 1993; Corder et al., 1993) there is still debate as to whether or not E-24.11 is an ECE. The identification of an ECE peptide was initially reported by Ohraka et al (1990). The peptide was membrane associated, inhibited by EDTA and EGTA chelators and insensitive to other proteases. Separate studies described the characterization of a membrane bound, Pr sensitive, thiorphan insensitive ECE which was distinct from E-24.11 and pH sensitive (Okada et al., 1990). Other studies reported similar findings describing a phosphoramidon sensitive cytosolic ECE metalloprotease (Matsumura et al., 1990, 1991). The lack of a specific assay which could combine sensitivity and speed of detection has delayed the purification of the ECE enzyme. The peptides initially purified were isolated from rat lung (Takahashi et al., 1993) and porcine aortic endothelium (Ohnaka et al., 1993). The rat enzyme was a 130,000 Mr protein (determined by SDS page) compared to the 90,000-100,000 Mr E-24.11 protein while the porcine aortic endothelium ECE was characterized as a 120,000 Mr protein (SDS page, Ohnaka et al., 1993). Two more sources of ECE activity were the bovine adrenal cortex (Mr 126,000) and transformed endothelial cell lines.

Purified ECE from membrane preparations of bovine endothelium cell line showed that ECE exists as a disulphide linked dimer with two 120,000 Mr subunits (Schmidt et al., 1994). Gel filtration of E-24.11 also revealed a dimer formation which is maintained by non-covalent interactions.

1.4.6 Molecular cloning of ECE

After the purification of ECE a number of groups cloned and sequenced the enzyme. ECE-1, which shows significant homology to E-24.11 and the erythrocyte blood group antigen, is a highly glycosylated transmembrane protein with a number of N-linked glycosylated sites and a highly conserved Cys rich domain. The N-terminal region shows great homology (>92%) between bovine, rat and human ECE enzymes (Schmidt et al., 1994). Variations between the other two bovine ECE enzymes may represent different isoforms. It is therefore possible that different subunits of ECE exist differing in the length of their cytoplasmic domains, possibly resulting from differential splicing. All ECE enzymes show a >95% homology in their extracellular domain amino acid sequences. ECEs are distributed broadly in a number of tissues such as the lungs, the pancreas, the placenta, the ovary, the testis and the adrenal gland. Endothelins are the only ECE substrates known so far. A number of studies have suggested possible sensitivity differences for the conversion of "big" ET-1 isoforms to the active peptides (Xu et al., 1994).

1.4.7 Endothelin receptors

Early ligand-binding studies using endothelins, suggested more than one ET receptor subtype (Kloog et al., 1989). Data accumulated by ligand binding and kinetic studies, chemical modifications of the receptors and cloning and expression of the receptor proteins reached similar conclusions. Two types of endothelin receptors have been identified so far and have been named ET_A-R (with higher affinity for ET-1 than ET-2 and lower affinity for ET-3) and ET_B-R (with equal affinities for all endothelin peptides). Both receptors have been cloned by different

groups (ET_A-R: Arai et al., 1990; Lin et al., 1991; Hosoda et al., 1992 and ET_B-R: Sakurai et al., 1990; Nakamuta et al., 1991; Ogawa et al., 1991). Binding studies using ^{125}I-ET radiolabelled ET's have identified high affinity (Kd's in the nM range) binding sites in brain, heart, lung, kidneys, liver, intestines, uterus, placenta, amnion and ovaries in the membranes of either isolated cell types, or tissue sections. Such studies indicated the co-existence and the differential distribution of ET_A-R and ET_B-R subtypes. Binding characteristics of the two receptor types in terms of Kds and Bmax values display significant variation between studies and can be caused in different ways. Firstly, individual studies have used different methods of tissue preparation including subcellular fractions or cell lines. However, differences may still be observed even when the same preparations were used, possibly due to alternative measuring techniques (Lewy et al., 1992). Secondly, variations in buffer composition or pH and the presence or absence of chelators such as Mg^{2+}, Ca^{2+} or BSA have proved important (Bolger etal., 1990; Maggi et al., 1991). Other critical factors included differences in the purity of the preparations, variations in the receptor concentration and in the ratio of the receptor to ligand and production of artifacts as the result of ligand becoming trapped within the cell when homogenate preparations were not used. Protection against degradation of the ligand and/or the receptors by a number of endogenous proteases required the presence of various protease inhibitors in the assay. In different species, different receptor subtypes may mediate the same function. In addition, differences between ET-R subtypes have been observed between species (Fukuroda et al., 1994b). Journeaux et al., (1994) demonstrated sex differences in the expression of ET-R in rats, glyconeogenesis being mediated by ET_A-R in males and by ET_B-R in females. Recently, a number of studies have suggested the existence of two ET_A-Rs and two ET_B-Rs, one with pM Kd values and one with nM Kd values in Rat-1, S3T3 fibroblasts (Ambar and Sokolovsky, 1993), and rat cerebellum (Sokolovsky et al., 1993). Two functionally distinct ET_A-R types were identified in rat mesangial cells (Simonson and

1993), and rat cerebellum (Sokolovsky et al., 1993). Two functionally distinct ET_A-R types were identified in rat mesangial cells (Simonson and Rooney, 1993) with Kds of 1.2 nM and 32 pM. Studies using the BQ123 ET_A-R antagonist to examine its effects on the binding of ET-1 to receptors in rat heart, have suggested that the inhibitory action of BQ123 was competitive at the nM sites but non-competitive at the p M sites (Sokolovsky et al., 1993), supporting the suggestion that BQ123 is an allosteric antagonist acting through a secondary site. The possibilities that these receptors can oscillate between the nM and pM affinity states, or that they represent fully different receptor subtypes have to be investigated. It has been suggested that although they represent two subtypes of similar Mr polypeptides (45-47 KDa), the pM receptor sites (ET_{B1}-R) are not coupled to the phosphoinositide hydrolysis signal transduction pathway while the nM sites (ET_{B2}-R) are and therefore induce the phospholipase C (PLC) activation (Mittler et al., 199). The same hypothesis is supported by data from Sokolovsky et al., (1994) and Shraga et al., (1994) which suggested that the pM sites are involved in the signal transduction via cAMP and cGMP pathways. Karne et al., (1993) demonstrated the presence of a third type of ET-R (ET_C-R) in *Xenopus laevis* with higher affinity for ET-3 and lower affinities for ET-1 and ET-2. The existence of such a receptor subtype could explain the fact that ET-3 and not ET-1 or ET-2 has been shown to mediate cell proliferation in wounded human umbilical vein endothelium. However, a study with rat brain capillary endothelial cells detected an ET_A-R type receptor sensitive to BQ123 showing high affinity for ET-3 (Sokolovsky et al., 1994).

1.4.8 Endothelin antagonists

In order to investigate further the potential role of endothelins in normal and pathological conditions, specific ET antagonists have been developed. Many ET antagonists which have been identified were discovered by screening natural products and compounds. The best example is BQ123, a highly selective ET_A-R antagonist, a cyclic ET-1 analogue pentapeptide

common antagonists. Most can be divided into two groups according to structure. The first group including derivatives of cyclic peptides (BQ123, RES-701-1, TAK 044) and the second groups derivatives of linear tripeptides (FR-139137, PD-151242, BQ788). The receptor antagonists bind to the receptor with high affinity (1-25 nM) and thereby inhibit the effects of ETs. Certain studies have shown that synergistic inhibition using BQ123 and BQ788 (ET_A-R and ET_B-R receptor antagonists) was needed for complete inhibition of the effects of ET-1 on the contraction of pulmonary arteries (Fukuroda et al., 1994a). Alternatively, non-selective ET receptor antagonists can produce simpler blockade (Clozel et al., 1994). Thus antagonists such as Bosentan (RO-0203) can bind to both ET_A-R and ET_B-R and block ET effects in isolated arteries (Clozel et al., 1994).

Antagonist	Structure	Receptor subtype	Reference
BQ-123	cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu)	ET_A	Ihara et al., 1992
PD 151242	N-[(hexahydro-1-azepinyl) carbonyl]-Leu (1-Me) D-Trp-D-Tyr	ET_A	Davenport et al., 1994
FR 139317	N-[(hexahydro-1-azepinyl) carbonyl]-Leu (1-Me) D-Trp-3-(2-Pyridyl)-D-Ala	ET_A	Nirei et al., 1993
50-235	27-0-caffeoyl myricerone	ET_A	Fujimoto et al., 1992
PD-142893	Ac-D-diphenylalanine-Leu-Asp-Ileu-Ileu-Trp	non selective	Cody et al., 1992
Ro46-2005	4-t-butyl-N-[6-(2-hydroxy-ethyoxy)-5-(3-methoxy-phenoxy)-4 pyrimidinyl-benzesulfonamide	non selective	Clozel et al., 1993
TAK-044	cyclo [D-α-aspartyl-3-[(4 phenyl-piperazin-1-yl) carbonyl] L-alanyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl]-disodium salt	non selective	Ikeda et al., 1994
RES-701-1	cyclic (Gly^1-Asp^9) (Gly-Asn-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp)	ET_B	Tanaka et al., 1994
IRL-1038	[Cys^{11}-Cys^{15}]-ET-1(11-21)	ET_B	Urade et al., 1992
BQ-788	N,Cis-2,6-dimethyl piperidinocarbonyl-L-α-methyl leucyl-D-1- methoxyl carbonyl-Trp-D-norLeu	ET_B	Ishikawa et al., 1994

Table 1.5: Structures and selectivities of the most common ET-receptor antagonists.

1.4.9 Molecular cloning/characterization of the receptors

cDNA clones encoding the two different subtypes of endothelin receptors have been isolated and characterised by a number of different groups. Data analysis of such studies revealed that the two receptor polypeptides have similar numbers of amino acid residues (415-442) and their molecular weights were estimated to be between 46,900 and 49,630. The homology between ET_A-R and ET_B-R in humans showed a 65% identity and sequences of ET_B-R between human, rat and bovine isoforms reached 85% homology. ET_C-R in *Xenopus laevis* consists of 424 amino acid residues with 47% and 52% homologies with ET_A-R and ET_B-R respectively (Karne et al., 1993). It is not yet clear if this receptor represents a variant of the two known types of ET receptors or a distinct ET_C-R. The same studies suggested that the amino acid sequences of ET receptors contain seven hydrophobic areas, each consisting of 22-26 amino acid residues which are separated by hydrophilic regions. This structure description is comparable with the G-protein coupled receptor complexes in which the polypeptide consists of seven transmembrane elements, an extracellular N-terminal domain and a cytoplasmic COOH tail. The N-terminus of all ET-R sequences contained a proline rich region although there was clear indication of major differences between the amino acid sequences of the two major ET-R subtypes. The N-terminus of ET_A-R consisted of 80 residues while that of ET_B-R consisted of 101 residues with a relatively limited homology. Further evidence as to the similarity of the receptors with the G-protein coupled receptors family is the presence of the Asp-Arg-Tyr triplet at residues 182-184 in ET_A-R and at 198-200 in ET_B-R (Lin et al., 1991). Finally, the different subtypes of ET-R might be due to alternative splicing of a single gene.

1.4.10 Kinetics of binding

The binding of ETs to specific ET receptors in various tissues has demonstrated a very slow dissociation of the ligand from the receptor, following binding. In some cases this process is almost irreversible and

dissociation rates vary between tissue types (Sokolovsky et al., 1994). The phenomenon could be explained as a dissociation of the ligand from a heterogeneous population of binding sites or in terms of a two step isomerisation model (Sokolovsky et al., 1989) with the dissociation rate varying depending on the pre-incubation period of the ligand with the receptors. Takasuka et al., (1994) suggested that the dissociation of ET_B-R-ligand complex was facilitated by proteolytic digestion during the signal transduction process.

1.4.11 Endothelin receptors and signalling

i) Phospholipase C (PLC) signal transduction pathway

It has been suggested that phosphoinositide (PIP_2) hydrolysis in human tissue, rat heart myocytes and rat cerebellar slices is mediated via two types of G-protein (a Gi or Go *Pertussis toxin* sensitive protein and a Gq type protein). The same studies have demonstrated that higher concentrations of endothelin are needed for stimulation of PLC activity compared to concentrations used for ligand binding studies. A number of reasons could account for this including the use of different experimental conditions for different experiments, multiple receptor subtypes, coupling of a limited number of receptors to PLC and existence of several different receptor-ligand complexes (Sokolovsky 1995). A study using Chinese hamster ovaries suggested that ET_B-R can activate two different signal transduction pathways depending on the concentration of ETs applied to cells (Etoh et al., 1994). In rat messangial cells the stimulation of the PLC pathway by ETs resulted in mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) concentration and activation of chloride channels (Iijima et al., 1991). The activation of chloride channels can , in turn, result in Cl^- efflux and further depolarization of the membrane. Such depolarization can cause the activation of voltage-dependent Ca^{2+} channels and therefore the increase in $[Ca^{2+}]_i$.

ii) Ca^{2+} Flux

In almost all systems, the binding of ETs to ET receptors results in Ca^{2+}

signalling which mediates a number of short-term effects such as vasoconstriction (Gulati and Srimmal, 1992; Maïani, 1993). It has been suggested that Ca^{2+} flux is mediated through two distinct mechanisms. The first is the IP_3 mechanism already discussed above, and the second is through the activation of multiple types of Ca^{2+} channels. It has been demonstrated that ETs can affect both voltage-operated and receptor-operated Ca^{2+} channels (which can be opened as the result of the involvement of second messengers). The addition of low doses of ET-1 in a number of systems stimulated a monophasic $[Ca^{2+}]_i$ increase which was sustained, and the duration of which was pronounced with ET-1 and ET-2.

iii) Phospholipase A_2 (PLA_2)

It has been reported that ETs stimulate PLA_2 in a number of tissues. Activation of PLA_2 lead to the release of arachidonic acid (AA) which was subsequently metabolised to prostaglandins (PG), leukotrienes and thromboxanes thought to act as second messengers mediating some of the biological activities of ETs (Reynolds, 1990; Resink, 1989). The observed release of arachidonic acid could be the result of two possible mechanisms which include either the direct activation of PLA_2 or the activation of an indirect pathway acting through PLC and DAG which could also lead to AA production. Studies have demonstrated the activation of PLC and PLA_2 in separate but parallel pathways (Schramek et al., 1994). Such activation of PLA_2 can occur following the binding of ETs to either ET_A-R or ET_B-R receptors. The same studies have demonstrated the activation of both PLA_2 and cytosolic PLA_2 by ET-1 binding to both such types of receptors (Schramek et al., 1994). ET-1 activated PLA_2 in a rapid manner which was regulated at the post-translational level. ET-1 also activated cytosolic PLA_2 in a much slower manner (Schramek et al., 1993, 1994).

iv) Phospholipase D (PLD)

PLD is a phospholipase which hydrolyses phosphatidyl choline and phosphatidyl ethanolamine (which constitute about 40% of all phospholipids) producing phosphatidic acid (PA) and choline or

phospholipids) producing phosphatidic acid (PA) and choline or ethanolamine. Recently, PLD activation by growth factors, neurotransmitters and hormones was shown to be a ubiquitous signal transduction pathway in mammalian cells (Dennis et al., 1991). ET-1 activated both PKC-dependent and PKC-independent PLD activation in Rat-1 fibroblasts (MacNulty et al., 1990). Similarly, PLD activation as a result of ETs biding to ET-Rs has been demonstrated in C6-glioma cells, Rat-1 cells and Swiss 3T3 fibroblasts, resulting in the production of DAG and PA with a number of potential effects such as prolonged maintenance of PKC activation. Brain is a rich source of PLD (Chalifour and Kanfer, 1980) as well as for ET_A-R. PLD activation can result in increased choline concentration and thus increased synthesis of acetylcholine in the brain. In the same way, phosphatidyl ethanol can lead to brain specific PKC activity as a substrate for phosphatidyl serine (Nishizuka et al., 1988).

v) Na^+/H^+ exchange

The regulation of Na^+/H^+ exchange has been linked with the binding of hormones, growth factors and other agents, to different receptors (Simonson, 1993). ETs were shown to activate Na^+/H^+ exchange in the brain (Vigne et al., 1991) through receptors with equal affinity for ET-1 and ET-3 and via a PKC independent mechanism. High ET (nM) concentrations may also result in the phosphorylation of Na^+/H^+ antiporter through a PKC activation (Simonson et al., 1989) while low ET concentrations (pM) regulate Na^+/H^+ exchange through a PKC-independent pathway (Kraker et al., 1990).

vi) cAMP/cGMP production

ETs regulate the production of cAMP in a number of systems. In some tissues such as in brain capillary endothelial cells, ETs activate cAMP formation (Ladoux and Frelin 1991) while in other systems, as in rat cardiac myocytes, they inhibit cAMP formation (Hilal et al., 1992). The effects of ETs are seen simultaneously on the PIP_2 hydrolysis and cAMP levels. Studies on the intracellular signal transduction pathways of ET_A-R and ET_B-R in Chinese hamster ovary cells (Aramori and Nakanishi,

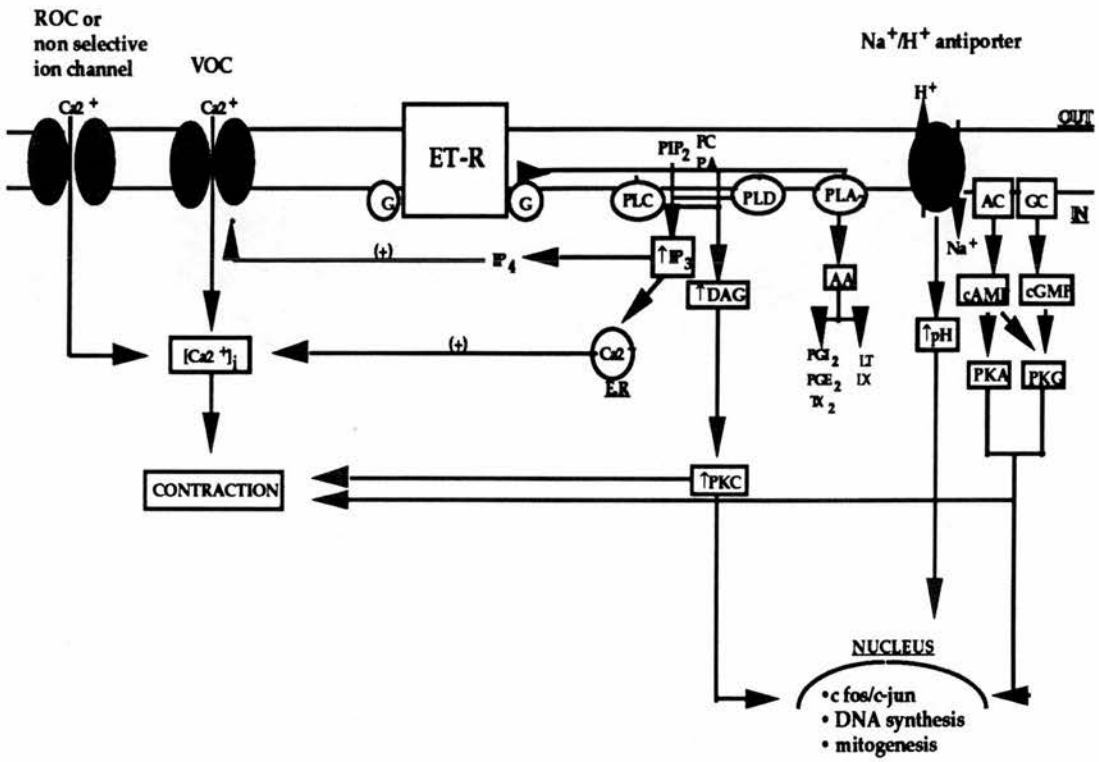
but cAMP formation was regulated by ET_A-R and cAMP inhibition was regulated by ET_B-R. However, such conclusions are not in accordance with studies in other systems and such diversity of results might shed light on the complexity of the signalling pathways involved. Sokolovsky et al., (1994) demonstrated that ET-1 at pM concentrations induces cAMP formation in a dose-dependent manner while ET-1 concentrations between 10^{-11}M and 10^{-7}M resulted in a dose-dependent inhibition of cAMP formation, reaching basal levels at 10^{-7}M ET-1. Such results could be explained by the presence of both main types of receptors (ET_A-R activation of cAMP and ET_B-R inhibition of cAMP) or by the possibility of a very fast desensitization process for the receptors as already demonstrated in rat myometrium (Khac et al., 1994; Cyr et al., 1993). cGMP synthesis is also stimulated by growth factors, hormones and neurotransmitters in various systems. Such stimulation can be mediated by nitric oxide (NO) binding to guanylyl cyclase which in turn stimulates the synthesis of cGMP (Moncada et al., 1991). In a number of tissues, ET-1 and ET-3 can mediate cGMP signalling at low doses as well as vasodilation caused by NO (Taniguchi et al., 1994). Increased stimulation of cGMP has been observed after the addition of ET-1 and SRTX-b at picomolar concentrations while when added at higher concentrations these same peptides caused a dose-dependent inhibition of cGMP. However, ET-3 and SRTX-c addition at picomolar concentrations did not stimulate cGMP formation while such effects were observed after the addition of the peptides at nanomolar or higher concentrations. These results suggest that the majority of the receptors are of the ET_B-R type while only a minority of the receptors are of the ET_A-R type and that the latter are responsible for the cGMP production. Further studies demonstrated that ET-1 and ET-3 activate cGMP formation through NO production and via a PT-sensitive G-protein(s) while SRTXs act via a different pathway which does not involve PT-sensitive G-protein(s). Therefore, ETs and SRTXs seem to bind to the same receptors and may produce the same second messengers but through different signalling

pathways.

viii) Signal transduction in the nucleus

ETs have also been shown to act through second messengers in order to activate MAP kinases and induce expression of genes such as c-fos, c-myc and c-jun (Simonson 1993). In chinese hamster ovary cells, (Tabuchi et al., 1994) ET_A-R and ET_B-R differentially activated the c-fos gene.

Figure 1.3 summarises the cell signalling pathways activated by endothelin.



AA:	arachidonic acid	AC:	adenylate cyclase
cAMP:	cyclic adenyl monophosphate	cGMP:	cyclic guanyl monophosphate
DAG:	diacyl glycerol	E.R:	endoplasmic reticulum
G:	G-protein	GC:	guanine cyclase
IP_3:	Inositol 1, 4, 5 tris-phosphate	LT:	leukotrienes
LX:	lipoxin	PA:	phospatidic acid
PC:	phosphatidyl choline	PGE2:	Prostaglandin E_2
PGI2:	Prostaglandin I_2	PIP_2:	phosphatidyl Inositol4,5 bis-phosphate
PKC:	Protein kinase C	PLA_2:	phospholipase A_2
PLC:	phospholipase C	PLD:	phospholipase D
ROC:	Receptor operated Ca2+ channel	TX_2:	Thromboxane 2
VOC:	voltage operated Ca^{2+} channel		

Figure 1.3: Diagrammatic representation of the cell signalling pathways activated by endothelin.

1.4.12 Endothelins and ovarian cancer

The role of endothelins in the ovaries and ovarian cancer has not yet been investigated in depth. The granulosa cells of the porcine ovary secrete (Kamada et al., 1991,1993) and human follicular fluids (Schiff et al., 1993) contain detectable amounts of immunoreactive ET-1. Human ovarian cancer cell lines SKOV-3, OVCA-433, A2780 and BG-1 produce substantial amounts of ET-1 (detectable by radioimmunoassay) (Bagnato et al., 1995). Even more limited information is available regarding the presence of ET-R in normal and malignant ovarian cells. In porcine ovarian granulosa cells, a single, high affinity (Kd: 0.59 pM) receptor type has been found, which has equal affinities for ET-1 and ET-3 (Kamada et al., 1991), suggesting that it was the ET_B-R subtype. In the ovarian carcinoma cell lines SK-OV-3, OVCA-433 and A2780 binding studies using radiolabelled ET-1 suggested the presence of a single type of high affinity ET_A-R. The addition of exogenous ET-1 to porcine granulosa cells (Kamada et al., 1993) and the human ovarian cancer cells mentioned above (Bagnato et al., 1995), resulted in a dose dependent stimulation of growth, suggesting a possible mitogenic role for ET-1 in these systems. Other effects such as increased $[Ca^{2+}]_i$ and IP_3 hydrolysis were observed as the results of exogenous addition of ET-1 to the cells, suggesting that the mitogenic effects were mediated by such changes.

1.4.13 Endothelins and breast cancer

The production of ET-1 from normal breast and breast cancer cells has been investigated. Baley et al., (1990) suggested that normal epithelial breast cells exhibited low constitutive expression of ET mRNA while normal breast stromal cells showed no expression of the same peptide. The production of ET-1 from breast cancer cells has been demonstrated in a number of lines, including MCF-7, BT-20, ZR-75-1 and ZR-75-30 (Kusuhara et al., 1990), MDA-MB-231, T47D, MCF-7 (Patel and Schrey 1995), epithelial cancer lines. In a study of breast tissue samples (Yamashita et al., 1991), it was shown that breast cancer tissue samples

contained 15 times more ET-1 than normal breast tissue samples and 6 times more than benign breast tumours. In the same study it has been shown that large amounts of ET-1 were present even at early stages of the disease (stage I) but there was no association between ET-1 concentration and prognostic factors such as tumour size, age of patients, and involvement of lymph nodes and it was thus concluded that ET-1 may be involved in the malignant transformation of breast ductal cells rather than the growth of cancer cells. It was also suggested that hormone dependent cell lines (MCF-7, T47D, ZR-75-1) produced up to 20 times more ET-1 than hormone independent breast cancer cell lines (MDA-MB-231, BT-20). In phyllodes tumours it has been shown that ET-1 is produced in even higher levels than other types of breast tumours (Yamashita et al., 1992). Two separate studies have suggested modulation of the production of ET-1 from breast cancer cell lines by molecules such as bombesin, cortisol, oestrogen, TNF-α, IL-1, IL-6 and TGF-ß. Bombesin, glucocorticoids and IL-6 have all been shown to stimulate the production of ET-1 in different cell lines of breast cancer (Schrey et al., 1992; Yamashita et al., 1993). In 1995, Patel and Schrey reported that human cancer cell lines (MCF-7, T47D, MDA-MB-231) contain a phosphoramidon-sensitive metalloproteinase which can process exogenously added big ET-1 to ET-1 (Patel and Schrey 1995). In breast cell lines and tissues, the presence of specific ET receptors has been associated with fibroblasts. Other studies have shown that ET-Rs are not present on the epithelial normal breast cells but associated with surrounding fibroblasts (Baley et al., 1990). Exogenous addition of ET-1 to S3T3 fibroblasts (Kusuhara et al., 1989), breast fibroblasts (Schrey et al., 1992) and other systems (Schichiri et al., 1991) produced mitogenic effects. ET-1 addition resulted in a modest increase in the incorporation of tritiated thymidine into human breast fibroblasts but these effects were enhanced with the addition of the peptide in the presence of IGF-1 (Schrey et al., 1992). The role of endothelins in breast cancer is still not fully defined but (i) increased production of the peptides from epithelial cancer cells

compared to normal cancer cells, and (ii) the absence of ET receptors in the epithelial cells but the presence of receptors in breast fibroblasts suggest a possible paracrine role for endothelins.

1.5 Aims

The aims of the present study were focused on expanding the present knowledge regarding the expression and roles for endothelins and their receptors in ovarian and breast cancer. More precisely this study aimed to:

i) measure the expression and secretion of endothelin by epithelial and fibroblast cell lines derived from ovarian and breast carcinomas.

ii) determine the expression of specific endothelin receptors and their binding characteristics in the same cell lines.

iii) monitor the effects of the exogenous addition of endothelin-1, -2 and -3 on the growth of these cell lines in culture.

iv) investigate the effects of the co-culture of epithelial and fibroblast cell lines on the growth of both cell types.

v) determine the expression of endothelins and endothelin receptors in a series of primary ovarian and breast cancers.

CHAPTER 2: MATERIALS/METHODS

2.1 MATERIALS

(In this section material suppliers are listed by technique.)

2.1.1 Cell lines

i) Epithelial cancer cell lines

The human ovarian epithelial cancer cell lines PEO4 and PEO14 were derived from the ascites of different patients (Langdon et al 1988). The PEO4 ascites was collected from a patient with a poorly differentiated primary serous adenocarcinoma who had been previously treated with chemotherapy while the PEO14 ascites was collected from a patient with a well differentiated primary serous adenocarcinoma prior to treatment.

ii) Fibroblastic cell lines

The human ovarian fibroblast cell lines (PEO9F, PEO12F, PEO14F and PEO27F) were cultured from the ascites of patients with ovarian cancer and the human breast fibroblastic cell lines (BRF1 and BRF2) were obtained from patients with breast cancer. S3T3 mouse fibroblasts were kindly donated by Dr H. Rozengurt, ICRF, London.

2.1.2 Tissue Culture

Alpha MEM	- Gibco, Paisley, UK
Cell scrapers	- Costar Ltd, High Wycomb UK
Dextran T70	- Pharmacia Biotech, St Albans UK
Dimethyl sulphoxide (DMSO)	- BDH-Merck Ltd, Leics UK
DMEM (with or without phenol red)	- Gibco
Foetal calf serum	- Advance Protein Products (Gibco)
Hydrocortisone	- Sigma Ltd, Poole, Dorset UK
Insulin	- Sigma
L-Glutamine	- Sigma
Microlance needles	- Becton/Dickinson, Oxford, UK
Multi-well plates	- Corning Inc., Staffordshire, UK
	- Falcon (Becton/Dickinson)
	- Nunc, Life tech., Paisley UK

Penicillin/streptomycin	- Gibco
Phenol red	- Flow laboratories ICN flow Buckinghamshire, UK
Phosphate buffered saline	- Oxoid-Unipath, Hampshire UK
Plastipak syringes	- Becton/Dickinson
RPMI with or without phenol red	- Gibco
Sodium selenite	- Sigma
Streptomycin	- Gibco
Tissue culture flasks	- Falcon, Nunc
Tissue culture inserts	- Falcon
Transferrin	- Sigma
Trypsin	- Gibco
Versene (EDTA)	- Sigma

2.1.3 Radioimmunoassay

Acetonitrile	- Rathburn chemicals Ltd Walkerburn, UK
C-18 cartridge columns	- Varian Ltd, Warrington UK
Cobra gamma counter	- Packard Cobra
Endothelin-1 (ET-1)	- Peninsula laboratories, Belmont, USA
Endothelin-2 (ET-2)	- Peninsula
Endothelin-3(ET-3)	- Peninsula
Methanol	- Prolabo (Merck)
RIA kit for the detection of Endothelin-1	- Peninsula
Trifluoroacetic acid	- Sigma

2.1.4 Receptor binding assays

i) Preparation of cell membranes

Benzamidine	- Sigma
EDTA	- Sigma
$MgSO_4$	- Sigma

Soybean trypsin inhibitor - Sigma

Tris/HCl - Sigma

Tris/maleate - Sigma

ii) Binding assay

BQ123 (ET_A receptor antagonist) - Peninsula

BQ788 (ET_B receptor antagonist) - Sigma

^{125}I-ET-1 - Amersham international

IgG immunoglobulin - Sigma

IRL1038 ET_B receptor antagonist - Peninsula

Polyethylene glycol - Sigma

iii) Protein concentration assays

NaOH - Sigma

Protein dye assay reagent concentrate - Bio Rad Ltd, Herffordshire, UK

Whatman number one filter paper - Whatman Ltd, Kent, UK

2.1.5 Growth assays

i) Antisense

Primers - ICRF Claire Hall, London, UK

ii) Co-culture

24 well-plate inserts - Falcon

Other materials as described in section 2.1.4

2.1.6 RT-PCR

(All chemicals and components from Promega unless otherwise stated)

i) RNA isolation

Chloroform - Fisons, Loughborough UK

Ethanol - Rathburn

Isopropanol - BDH

Tri reagent^{LS} - Research Centre Inc. (Promega)

Tri reagent^{TM} - Research Centre Inc.

ii) RT-PCR

Agarose - Gibco BRL

Primers - ICRF Claire Hall

Reverse transcriptase - Gibco BRL

Taq polymerase - ICRF Claire Hall

Thermal cycler - Techne, Cambridge, UK

100 bp DNA ladder - Gibco BRL

2.1.7 Southern blotting

γ^{32}P-ATP - Amersham

DNA 5′ end labelling system - Promega, Southampton, UK

N+ membrane - Boehringer Mannheim, East Sussex, UK

Probes (oligonucleotides) - ICRF Claire Hall

2.1.8 Immonohistochemistry

Avidin biotin peroxidase - Dako Ltd, Wycombe, Bucks, UK

3,3′ diaminobenzidine - Sigma

DPX synthetic mounting medium - Fisons

Epithelial common antigen -Dako limited

ET-1 human monoclonal antibody - Peninsula

ET-2 human monoclonal antibody - Peninsula

ET-3 human monoclonal antibody - Peninsula

Fibroblast antigen - Dako limited

Hydrogen peroxide - Sigma

Leukocyte common antigen - Dako limited

Rabbit anti mouse biotinylated antibody - Dako limited

Tris buffered saline - Sigma

Xylene - Fisons

2.2 METHODS

2.2.1 Cell culture

i) Growth of cell lines

The breast epithelial cancer cell lines MDA-MB-231, T47D, ZR-75-1, the ovarian adenocarcinoma cell lines PEO4, PEO14 and the S3T3 mouse fibroblast line were routinely cultured at 37°C in an atmosphere of 5% CO_2, 95% air (90% humidity) in Dulbecco's Modified Eagle Medium (DMEM) containing phenol red pH indicator. The medium was supplemented with 10% FCS (v/v), L-glutamine (2mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The ovarian fibroblastic cell lines PEO12F, PEO14F, PEO27F were cultured under the same conditions, also in DMEM containing L-glutamine, penicillin and streptomycin but supplemented with 15% FCS. Breast cancer fibroblastic cell lines, code-named BRF1 and BRF2, were routinely cultured in Alpha Modified Eagle Medium (Alpha MEM) supplemented with 15% FCS and L-glutamine, penicillin and streptomycin.

ii) Subculturing

Monolayers of all the cell lines were grown to confluence in 75 or 125 cm^2 flasks. Media were discarded and the cells were washed twice with phosphate buffered saline (PBS) pH 7.3. Cells were then detached from the plastic by incubating with trypsin (0.25% (w/v) in Gibco solution) / versene (1mM EDTA in PBS, 0.5% (v/v) phenol red) solution (1:1) for 5-15 min at 37°C. Following trypsinisation, 5 ml of media were added to the flasks to neutralize the effect of trypsin and cells were collected in a Universal container. A microlance 3 needle (0.8x40 mm, 21G for PEO4, MDA-MB-231, T47D, ZR-75-1, S3T3 and 1.1x40 mm, 19G for PEO14, PEO12F, PEO14F, PEO27F, BRF1 and BRF2 cell lines) was used to syringe cells and create single cell suspensions. Aliquots of cells were seeded into new flasks. For routine culture, media were replenished every 3-4 days and cells were passaged once a week.

iii) Preservation and resuscitation of cells from liquid nitrogen

Stocks of each cell line were created by growing cells to near confluence

(80-90%), trypsinising and collecting in a Universal container. An aliquot (1 ml) of cell suspension was counted in the haemocytometer to estimate total cell count. The remaining cells were washed with 5 ml of fresh media containing serum and centrifuged at 1000 x g for 5 min. Aliquots of the remaining pellet were re-suspended in ice cold 10% (v/v) dimethyl sulphoxide (DMSO) in foetal calf serum (FCS) at a concentration of $5x10^6$ cells/ml and transferred into cryotubes. Samples were kept at -80°C for 24 h before being stored in liquid nitrogen for future use. Cells were resuscitated by removing from the liquid nitrogen and, in order to thaw rapidly, immersed in a 37°C warm water bath. Cells were then washed with 5 ml of DMEM media containing FCS (percentage depending on the cell line) and centrifuged at 1000 x g for 5 min. The cell pellet was re-suspended in fresh media (5 ml) and transferred to a 25 cm^2 flask. After 24 h in culture, cells usually had attached firmly to the surface of the flask. Attached cells were washed with PBS and covered with fresh media (5 ml).

2.2.2 Radioimmunoassay of conditioned media for endothelin

i) Preparation and collection of conditioned media

For the preparation of conditioned media for the radioimmunoassay, each cell line was subcultured in 3 x 25 cm^2 flasks and allowed to grow to 90% confluence in phenol red containing media supplemented with the standard amount of serum. Cells were then washed twice with 5 ml of pre-warmed PBS and incubated in 10 ml phenol red- and serum-free DMEM supplemented with HITS (10nM hydrocortisone, 5µg/ml insulin, 10µg/ml transferrin, 30nM sodium selenite). After 24h this was removed, fresh media were added and cells were incubated for a further 72 h. The resulting conditioned media were collected and the cells in each flask were trypsinised and counted.

ii) Extraction

Conditioned media collected from different cell lines were applied to C-18 cartridge columns which had been previously activated with 3 x 1ml

methanol and washed with 3 x 1ml distilled H_2O. The columns were then left at room temperature for 60 min and the bound material was eluted with 3 ml of 60 % acetonitrile, 0.1% trifluoroacetic acid (60% ACN, 0.1% TFA) and collected in glass tubes. The eluents were evaporated down overnight using a Univap Uniscience centrifuge and stored at 4°C until assayed.

iii) Radioimmunoassay

The presence of immunoreactive ET-1 in the conditioned media of different cell lines was investigated using a commercially available radioimmunoassay kit (Peninsula). Samples and standards in the RIA were prepared in duplicate and each experiment was performed on 3 occasions. For the preparation of the standards in the radioimmunoassay, 100 µl of increasing concentrations (1, 2, 4, 8, 16, 32, 64, 128 pg/100µl) of standard ET-1 were added (in duplicate) to 12 x 75 mm plastic tubes. Three extra pairs of tubes were prepared TC-1 and 2 (total counts), NSB-1 and 2 (non-specific binding), TB-1 and 2 (total binding) with no RIA buffer (TC tubes) and 200 µl of RIA buffer (NSB and TB tubes) respectively. Samples of each conditioned media were diluted, 1:1, 1:2, 1:4 and 1:8 and aliquots (100 µl) of these dilutions were added to the tubes in duplicate. Primary rabbit anti ET-1 serum (100 µl) was added to TB and all standards and samples. The tubes were mixed by vortexing, covered in foil and incubated for 24 h at 4°C. At the end of the incubation period, 100 µl of 15,000 cpm ^{125}I-ET-1 was added to all the tubes, mixed and incubated for a further 24 h at 4°C. At the end of this second incubation period, goat anti-rabbit IgG (100 µl) and normal rabbit serum (100 µl) were added to all tubes. Samples were mixed by vortexing and incubated at room temperature for 90 min. RIA buffer (500 µl) was then added to all tubes, mixed and centrifuged at 1700 x g for 20 min at 4°C. The supernatant was then aspirated from the tubes (apart from TC tubes) with an elongated, fine point pastette and radioactivity in the tubes was counted using a Cobra gamma counter. TB tubes demonstrated the "maximum" binding of the assay while NSB tube accounted for the non-specific binding value

in the assay. From the counts of the standards in the assay, a plot was drawn of the %specific binding (%B/Bo) against the log of the standard concentration of the ET-1-like peptide measured in the assay. From the resulting sigmoid curve and with the use of the %B/Bo value obtained for each sample in the assay, it was possible to calculate the concentration of the ET-1-like peptide for each sample, by finding the intersection point with the curve and then reading the corresponding X-axis value. Values were multiplied by the dilution factor and mean values were obtained for each individual sample.

2.2.3 Receptor binding assay

i) Preparation of cell membranes

Cells were grown to 70% confluence in 125 cm^2 flasks in DMEM media supplemented with 10% FCS and subsequently cultured in serum-free DMEM media supplemented with HITS until fully confluent. Cells were then trypsinised with 2ml of trypsin/versene mixture and collected by centrifugation at 1,000 x g for 10 min at 4°C. The cell pellet was re-suspended in buffer-1 (10mM Tris/HCl, 1.5 mM EDTA, 1 mM benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4) sonicated using a SONIPREP MSE sonicator and re-centrifuged at 1,000 x g for 5 min at 4°C. The resulting supernatant was ultracentrifuged at 100,000 x g in a Beckman TL 100 for 30 min at 4°C and the pellet re-suspended in 0.5 ml of buffer-2 (50mM Tris/maleate, 10mM $MgSO_4$, 1mM benzamidine, 0.002% soybean trypsin inhibitor, pH 7.4). The "membrane" fractions were passed through a pastette to disaggregate clumps, transferred into cryotubes and stored at -80°C, until assayed. An aliquot (100 ml) of the membrane preparation was stored for protein estimation at a later date.

ii) Binding assay

The binding assay was performed in 12 x 75 mm Pyrex borosilicate glass tubes. Duplicate aliquots (2 x 100 µl) of membrane preparations were incubated with 50µl of increasing concentrations of non radioactive ET-1 (final concentrations of: 50, 100, 200, 450, 950, 1450, 2950, 4950, 6950, 9950

pM) added to 50µl of a fixed (50 pM) concentration of ^{125}I-ET-1. The radiolabelled and standard ET-1 were left for 10 min at 30° and a membrane fraction (100 µl) was added to each tube. Samples were placed in a shaking water bath for 90 min at 26°C; conditions found to be optimal for this assay (results section). To terminate the reaction, tubes were placed on ice and 0.5 ml of ice-cold 0.5% (w/v) immunoglobulin G (IgG) was added to each tube, followed by 1 ml of 12.5% (w/v) polyethylene glycol (PEG). Separation of the bound and free ^{125}I-ET-1 was performed by centrifugation at 3000 rpm for 15 min at 4°C using a swing out rotor on a Beckman CPR centrifuge. After centrifugation, samples were placed on ice and the supernatant, containing the free ^{125}I-ET-1, was aspirated with a fine-tip pastette and the remaining pellet, containing the bound ^{125}I-ET-1, was counted in a Packard Cobra Gamma Counter. The "total" cpm was calculated using two tubes containing the same amount of labelled ET-1 (50 pM) but no membranes or standard ET-1. Non-specific binding (NSB) was also calculated using 1µM of standard ET-1 and was found to be less than 10% of total binding.

iii) Receptor site concentration

The cpm value of ^{125}I-ET-1 in each tube, was corrected by subtraction of the value for the NSB and data were analyzed and plotted according to the method described by Scatchard (Scatchard, 1949). For interpretation of the data, the "Ligand" computer program (Biosoft) was used to determine dissociation constant values (Kd) and x-axis intercept values (Bmax). The same programme was used in the case where two components were present in the graph, to fit, assign and calculate, the Kd and Bmax values.

iv) Binding inhibition experiments

For the binding inhibition experiments the same protocol as described above was used. Aliquot of cell membrane samples (in triplicate) were incubated at 26°C for 90 min with ^{125}I-ET-1 (50pM), increasing concentrations (10^{-13} to 10^{-6}M) of ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). At the end of the incubation period specific binding was calculated as described previously. Non-specific

binding was determined in the presence of 1μM ET-1.

v) Protein concentration assays

A modified version of the Bradford assay (Bradford et al 1976) was used to measure the protein concentration of the membrane preparation. Aliquots (100 μl) of (i) BSA standards (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg/ml), (ii) two BSA quality controls (0.4 and 0.8 mg/ml) (iii) the membrane preparations which had been incubated with 2M NaOH (100 μl) for 1 h at 60°C and neutralised by the addition of 2M HCl (100 μl), were prepared. For each membrane preparation dilution sample (20 μl), BSA standard, quality control sample, or simple buffer sample triplicate 12x75 mm glass tubes were prepared. The reagent solution was prepared by diluting Bio-Rad Protein Dye Assay Reagent Concentrate 1:5 in distilled water and filtering through Whatman Number One filter paper. This solution was added (1 ml) to the last solution and mixed by vortexing. Aliquots (200 μl) of each solution were pipetted into 96-flat bottomed well microtitre plates and the optical densities (OD) of the solutions were read in a Bio-Rad automatic plate reader at 595 nm. The protein concentration of each sample was calculated by comparison of the sample's OD with those on the standard curve.

2.2.4 Growth assays

i) Growth curves

Cells were plated in 24 well plates at a concentration of 2.5 x 10^4 cells/ml and left to grow for 9 days in the conditions optimal for each cell line (see section 2.2.10) or in 1% FCS. Quadruplicate wells were trypsinised and counted on days 0, 2, 4, 7, 9 and 11.

ii) Preparation of HITS nutrient

The preparation of HITS nutrient was performed in sterile conditions by dissolving insulin (5 μg/ml) in 0.01M of NaOH/EtOH in a total volume of 1L. To the resulting solution, hydrocortisone, transferin and sodium selenite were added. The HITS stock solution was stored in aliquot (5 ml). One aliquot was added to each 500 ml of media (final concentrations of

nutrients: hydrocortisone 100 nM, insulin 5 μg/ml, transferin 10μg/ml, and sodium selenite 30 nM).

iii) Effects of ETs on the growth of cell lines

The effects of the exogenous addition of ETs on the growth of different cell lines were investigated as follows. The growth curves for the cell lines were determined in DMEM + 10% FCS, DMEM + 15% FCS and in Alpha MEM + 15% FCS (breast fibroblasts) and phenol red-free DMEM +1% FCS and phenol red -free Alpha MEM + 1% FCS. For MDA-MB-231, T47D, ZR-75-1, PEO4, PEO14, S3T3 cell lines, cells were harvested in mid-log phase as mentioned before (see section 2.1.2) and sub-cultured in 24 well plates (0.5 ml/well) at a concentration of $5x10^4$ cells/well ($2.5x10^4$ cells/well for MDA-MB-231, T47D, ZR-75-1 and PEO4 cells), using an Eppendorf multidispenser. Cells were cultured at such conditions for 24h and were subsequently washed x2 with pre-warmed PBS (0.5 ml/well) and incubated in phenol red-free DMEM + 1% FCS for a further 24h. Phenol red-free DMEM was used because it has been shown (Berthois et al 1986) that phenol red possesses weak oestrogenic activity. Fresh 1% FCS +DMEM with or without concentrations of ETs ranging from 10^{-6}M to 10^{-13}M were added to the cells (Day 0); cultures were incubated for 48h, at the end of which period the same addition was performed (Day 2) and cells were incubated for a final 24 h. Finally (Day 5), cells were washed twice again with pre-warmed PBS, were trypsinised in 250μl trypsin/versene mixture/well and counted in a Coulter counter. For PEO12F, PEO14F and PEO27F ovarian fibroblasts the same method was used but cells were initially plated in 15% FCS DMEM instead of 10% FCS. In the case of breast fibroblasts, cells were cultured in 6 well plates because of their larger size and tendency to be contact inhibited. They were initially plated in 2 ml of 15% FCS/Alpha MEM media, were washed in 2ml of pre-warmed PBS and were trypsinised using 1 ml of trypsin/versene mixture. ET additions and replenishment of the media were performed as before and at the same time-points as for the other cell lines. Each set of conditions was studied in quadruplicate and in some cases cells were

counted on days 0, 2, 5, while in others, cells were counted only on day 5. For the receptor antagonist experiments, the same protocol was used but 100nM of either BQ123 (ET_A-R) or BQ788 (ET_B-R) antagonists was added to cells at the same time as ETs of fresh media.

iv) "Antisense" experiments

For the "antisense" experiments, specific "antisense" oligonucleotides against ET_A and ET_B receptors mRNA were designed to block the translation of these RNA molecules to proteins. Antisense oligonucleotides were targetted to the start codon (methionine) within the mRNA human ET_A-R (Sakamoto et al., 1991) and ET_B-R (Elshourbary et al., 1993) sequence. "Sense" oligonucleotides for ET_A and ET_B mRNAs as well as a "random" oligonucleotide were also designed to test the specificity of the "antisense" and their sequences are shown in table 2.1. PEO4 and PEO14 cells were seeded in 24 well plates at concentrations of 2.5 and $5X10^4$ cells/well respectively and cultured in 10% FCS for 24h. Old media were then replaced with serum free media (DMEM+HITS) and after a further 24h, concentrations of 10 or 30μM of "antisense", "sense" and "random" oligonucleotides were added to cell cultures. After a further 48h, fresh oligonucleotides were added and cells were cultured for two days, at the end of which period they were trypsinised (as described before) and counted using a Coulter Counter.

Oligonucleotide	**Sequence (5′ to 3′)**
ET_A-R sense	ATGGAAACCCTTTGC
ET_A-R antisense	GCAAAGGGTTTCCAT
ET_B-R sense	ATGCAGCCGCCTCCA
ET_B-R antisense	TGGAGGCGGCTGCAT
random	GAACTCGAGGTTCAT

Table 2.1: Sequences and general information on oligonucleotides used in anti-sense experiments.

v) Co-culture experiments

For the co-culture experiments, fibroblasts (PEO12F and BRF1 cells) were

seeded in wells of 24 well-plates ($5x10^4$ cells/well) at standard serum (10% and 15%, respectively) conditions, while epithelial cancer cells (PEO14 and T47D cells) were seeded (at $2.5x10^4$ cells/well in 10% serum) in 24 well-plate inserts that were placed in separate wells. After 24h, epithelial cells, contained in well inserts were transferred on top of fibroblasts cells and were cultured together for 5 days in 1% serum in the presence or absence of BQ123 or BQ788 ET-receptor antagonists (100nM). At the end of the incubation period cells were trypsinised and counted using a Coulter Counter.

vi) Coulter counting

At the end of the 5 day incubation period, media were removed from each well, cells were subsequently washed in warmed PBS (0.5 ml/well) and detached from the well by incubation with trypsin/versene mixture (0.25 ml/well) at 37°C for a few minutes. Detachment of the cells from the well was observed under the microscope and cells were passed through a microlance 3 syringe (0.8x40 mm, 21G for PEO4, MDA-MB-231, T47D, ZR-75-1, S3T3 and 1.1x40 mm, 19G for PEO14, PEO12F, PEO14F, PEO27F, BRF1 and BRF2) to obtain single cell suspension. An aliquot of the cell suspension (200µl) was added to 0.9% NaCl (9.8 ml) and gently mixed. Each suspension was counted 3 times using a 200µm diameter probe in a ZF Coulter counter. In order to calculate number of cells /well the mean value of the triplicate counts were multiplied by the dilution factor (x 25 for the 24 well plates and x 100 for the 6 well plates).

2.2.5 RNA ISOLATION

i) Homogenisation of tumours

Tumour samples were removed from liquid nitrogen, placed in a Falcon 35 x 10 mm petri dish and sliced into small pieces, as rapidly as possible, with a sterile surgical blade under sterile conditions. Pieces of tumour were then placed into a homogenisation capsule of a MIKRO-DISMEMBRATOR II homogeniser and homogenised for ten seconds or until completely powdered. The tumour powder was subsequently

removed from the capsule into a SARSTEDT (95 x 16.8 mm) centrifuge tube using a pastette and TRI REAGENT™ was added to the tumour preparation (1 ml per 50-100 mg of tissue). Tumour homogenates were stored at -80°C before RNA extraction.

ii) RNA isolation from tumour homogenates

Homogenized samples were left for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes. Chloroform (0.2ml/1ml) of TRI REAGENT™) was added to each tube which was then covered tightly, vigorously shaken for 15 s and left at room temperature for 15 min. The resulting mixtures were centrifuged at 12,000 x g for 15 min at 4°C using a Sorvall RL-5B (DU POND) centrifuge. After centrifugation, the mixture separated into three phases. The red-coloured phenol-chloroform lower phase, contained the proteins, the white interphase contained most of the DNA and the upper clear aqueous phase contained the RNA. The clear aqueous RNA phase which constituted 60% of the total volume, was removed into new tubes. For precipitation of RNA, isopropanol (0.5 ml/1 ml of TRI REAGENT™) was added to each tube. At this point samples were dispensed into 1 ml eppendorf tubes, left at room temperature for 10 min and subsequently centrifuged at 12,000 x g for 10 min at 4°C using an Eppendorf centrifuge. At the end of the centrifugation, the RNA precipitate formed a gel-like pellet on the side and bottom of each eppendorf tube. The supernatant was removed using an extended nose pastette and pellets were washed with 75% ethanol (1 ml of 75% ethanol/ 1 ml of TRI REAGENT™) by vortexing and centrifuged at 7,500 x g for 5 min at 4°C (Eppendorf centrifuge). The supernatant was removed with an extended nose pastette and the pellet was dissolved in 40 µl of diethyl pyrocarbonate (DEPC) treated water. A small aliquot was removed (5 µl) for concentration assessment and the remaining RNA was stored at -80°C for future use.

iii) RNA isolation from cell lines

For the preparation of RNA from cell lines, cells were allowed to grow to confluence using routine culture conditions in 75 cm^2 flasks. Media were

removed using a sterile 25 ml plastic pipette and TRI REAGENT^{LS} (1 ml/10 cm^2) was added to each flask in order to lyse the cells. The lysate was passed through the pipette several times to allow complete lysis and was transferred into a SARSTEDT (95 x 16.8 mm) test and centrifuge tubes. For RNA isolation, the same protocol described above (section 2.2.5) was followed.

The yield and purity of the RNA preparation products were measured by mixing the RNA preparations (5µl) with water (1295µl) and measuring the light absorbance of the mixture at wavelengths of A_{260} and A_{280} in a UNICAM UV/VIS spectrophotometer. Samples with very low yield (<5 µg) and/or low purity were discarded (purity = A_{260}/A_{280} and the acceptable values are 1.6-2.0).

2.2.6 RT-PCR

i) Selection of oligonucleotide primers

The human ET-1, ET-2, ET-3 DNA gene sequences as published by Bloch et al 1989, 1991 were used in order to design specific primers for all 3 endothelin peptides with the assistance of the Gene Jockey program. The primers used for ET_A-R, ET_B-R and ß-actin were originally designed by Pekonen et al (1995). The sequences of all the primers are shown in table 2.2.

RNA	Oligonucleotide sequence	Nucleotide number	Product size (bp)
ET-1	5' primer: ATGGATTATTTGCTCATGATTTT 3' primer: CTTGGGATCATGAAAAGATGATTT	268-291 826-849	582
ET-2	5' primer: AGGAAGAGATAGTGTCGTGAGC 3' primer: ACACAGAACTGCCTTGGACGT	596-617 773-792	197
ET-3	5' primer: TGTGAGTGTGGAGATGTTATCC 3' primer: TTCTCTCTGATACCATCTTGCC	1662-1683 2134-2154	493
ET_A-R	5' primer: CACTGGTTGGATGTGTAATC 3' primer: GGAGATCAATGACCACATAG	38-57 386-405	368
ET_B-R	5' primer: TCAACACGGTTGTGTCCTGC 3'primer: ACTGAATAGCCACCAATCTT	308-368 818-837	530
ß-actin	5' primer: CCCAGGCACCAGGGCGTGAT 3' primer: TCAAACATGATCTGGGTCAT	154-173 396-415	262

Table 2.2: Oligonucleotide sequences and general information on primers used in PCR amplification.

ii) Reverse transcriptase

For reverse transcription of the endothelins, their receptors and ß-actin, a small aliquot of each RNA (5 µg) prepared from cell lines and tumour samples was mixed with 1 µl oligo dT primer, 2 µl TaQ buffer (50 mM KCl, 10 mM Tris, 0.1%TritonX-100), 2 µl of each deoxy-NTP (1 mM) and 4 µl $MgCl_2$ (5 mM). The mixture was heated to 65°C for 5 min in a Techne PHC-3 programable thermal cycler for the RNA to anneal to oligo-dT primers. The mixture was then transferred onto ice for 2 min before RNAsin (0.5 µl) and reverse transcriptase (RT) (0.5 µl) enzymes were added to each tube. Samples were re-placed in the thermal cycler at 42°C for 1 h to allow the first strand cDNA synthesis. This was followed by incubation for 1min at 92°C to inactivate the RT enzyme.

iii) Preparation of the primers

All the PCR primer oligonucleotides were received in eppendorf tubes. To each primer, 60 µl of sodium acetate (1 M), 80µl $MgCl_2$ (25 nM), 60 µl of dH_2O and 600 µl of cold ethanol were added. The oligonucleotides were left on dry ice for 15 min and centrifuged at maximum speed (15,000 rpm) using an eppendorf centrifuge for 5 min. The supernatant was removed using a pastette and pellets were washed in 1 ml of 80% cold ethanol. Primers were mixed and re-centrifuged once again at maximum speed for 5 min. The ethanol was removed and pellets were re-suspended in 1 ml of RNAse-free H_2O and stored at -40°C. A small aliquot (10 µl) of each primer was re-suspended in 990 µl of H_2O.

iv) Polymerase chain reaction (PCR)

For the PCR reaction, 1 µg of reverse transcribed RNA product (4 µl) was added to a total of 20 µl of PCR mixture containing 0.3 mM dNTP (0.6 µl), 10 pmol of each primer (0.6 µl) and 2.5 units of Taq polymerase (0.5 µl) in buffer containing 1.875 mM $MgCl_2$, 10 mM Tris, 50 mM KCl, 0.1% Triton X-100 and RNAse free water. The reaction mixtures were set up in Eppendorf tubes and mixed. A drop of light mineral oil was added to each tube for the prevention of evaporation of the mixture. The PCR reaction was a three step reaction incubating at 93°C (1 min), 57 or 60°C (1

min) and 72°C (1 min); products were amplified for 32 cycles at the above temperatures followed by a further 5 min run at 72°C. The products of the reaction were visualised under UV light with the use of ethidium bromide on a 1.4% agarose gel which ran for 90 min at 100V.

2.2.7 Southern Blotting

i) DNA Transfer

A photograph of the gel of the PCR products was taken and the position of each band on the gel noted before immersing the gels in distilled water for 30 min for destaining. Capillary action was used to transfer the DNA from the gel onto a nylon membrane overnight using an alkaline transfer (0.4 M NaOH). After the completion of the transfer the membrane was air dried, sealed in a plastic bag and stored at 4°C until used. To check for the complete transfer of the DNA on the membrane, the gel was re-stained in ethidium bromide solution for 30 min and visualised under UV light.

ii) Probe labelling

The oligonucleotides used for the probe labelling were designed using the cDNA sequences of the 3 endothelin isoforms and the two receptor types as published (table 2.3). The specific sequences were contained within the section of the cDNA sequence of the selected primers used for the PCR amplification. The oligonucleotides were end-labelled with γ^{32}P ATP in a reaction mixture containing 20 ng of oligo, 3 units of T4 polynucleotide kinase, in 1x forward exchange buffer and 1.85 mBq of γ^{32}P ATP. The mixture was mixed and incubated for 30 min at 37°C.

Oligonucleotide	Sequence (5′ to 3′)
ET-1	GCACGTTGTTCCGTATGGACTTGG
ET-2	GACTCTCTGCCTGCTTCCTGGACC
ET-3	ATTTATTGTGAAACTGTTCTCCAC
ET_A-R	GCACAACTATTGCCCACAGCAGAC
ET_B-R	TGTGTAAGCTGGTGCCTTTCATAC

Table 2.3: Sequences of oligonucleotides used in Southern blot experiments.

iii) Hybridisation and washing

Membranes were submerged in 6x saline sodium citrate (SSC). In order to remove any air bubbles, the membranes were carefully rolled between two pieces of nylon gauze. The membranes were then placed in hybridisation oven tubes, immersed in hybridisation solution (5x SSc containing 0.1% SDS, 0.1% sodium pyrophosphate, 0.05% BSA, 0.05% polyvinyl pyrolidine, 0.05% ficol) and prehybridised for 30 min at 48°C. The solution was poured off and 25 ml of hybridisation solution containing the labelled oligonucleotide was added. The membranes were left in this solution for 4h at 48°C after which period filters were rinsed twice and washed four times at 48°C for 15 min in 4x SSC, 0.1% sodium pyrophosphate. The filters were then sealed in plastic bags and autoradiography was carried out at room temperature, where filters were exposed for 30 min to 2h.

2.2.8 Immunohistochemistry

i) Tumour samples

Paraffin fixed sections of tumour samples were dewaxed in xylene for 10 min and washed in descending concentrations of ethanol before being placed in 1% hydrogen peroxide for 15 min and washed in water. The sections were then washed in Tris buffered saline (TBS) for 5 min and were incubated in a moist environment with 20% FCS in TBS for 10 min. Excess TBS/FCS was drained off and sections were covered with primary human ET-1 (1: 200 dilution in water), ET-2 and ET-3 (1:400 dilution in water) antibodies for 30 min at room temperature followed by 2x 5 min washes with TBS. The sections were covered with avidin biotin peroxidase (AB) complex dissolved in TBS for 20 min at room temperature and washed as before. The sections were then covered in 1 mg/ml solution of 3,3′ diaminobenzidine (DAB) containing 5% hydrogen peroxide for 5 min at room temperature and washed in water. Finally, the sections were counterstained lightly in haematoxylin, dehydrated in alcohol, cleared in xylene and mounted in DPX under coverslips.

ii) Multispot slides

For the preparation of the multispot slides from the ovarian fibroblast cell lines, cell suspensions (20µl of $5x10^6$ cells/ml) were added to each well on the slides. Multiwells were left to dry overnight and then fixed in 1:1 acetone/methanol solution for 10 min. Fixed slides were left to air dry and transferred to -20°C until used. Multispot slides were processed further using the same protocol as the tumour samples, discussed above. Four primary antibodies were added to the slides: monoclonal mouse anti-human fibroblast (1:10 dilution), epidermal membrane antigen (1:40), common leucocyte antigen (1:10) and human endothelin 1 (1:200).

2.2.9 Statistics

All the statistical analysis were based on paired t-test analysis of variant.

Chapter 3: Cell lines and growth

The role of the endothelin family in ovarian and breast cancer was studied using a number of cell lines as models. Two human ovarian cancer (PEO4 and PEO14), three breast cancer (MDA-MB-231, T47D and ZR-75-1), three human ovarian fibroblast (PEO12F, PEO14F and PEO27F) and two breast fibroblast (BRF1, BRF2) cell lines were used in this study. The growth of these cell lines in varying serum conditions was first established.

3.1 Ovarian cell lines

3.1.1 Ovarian epithelial cancer cell lines

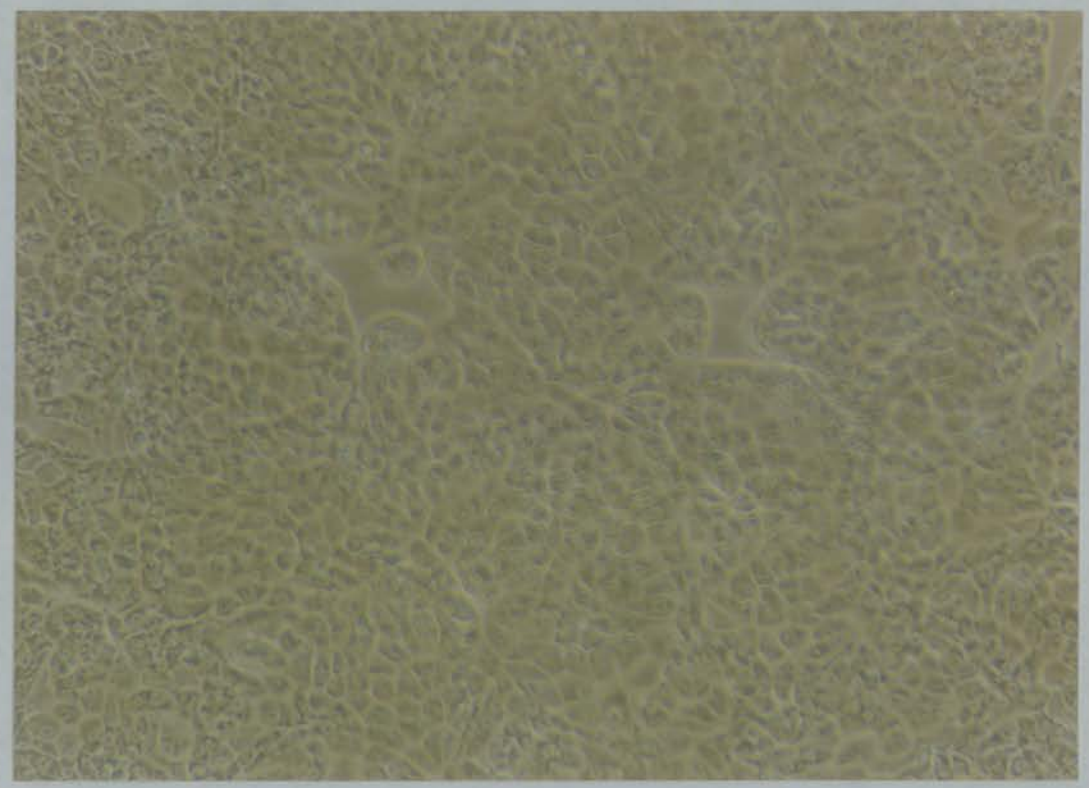
The two cancer cell lines, PEO4 and PEO14, were routinely cultured in 10% serum and images of the cultures of these cells are shown in photographs 3.1 and 3.2. The growth of these cells in DMEM culture media supplemented with 10% (with phenol red) or 1% (without phenol red) foetal calf serum (FCS) over an 11 day period is discussed below.

i) PEO4 cells

PEO4 cells were seeded at a concentration of 2.5×10^4 cells/well in 24 well plates. Cells growing in 10% FCS DMEM progressively increased in number over the culture period, reaching 50×10^4 cells/well at the end of the 11 day incubation. In contrast, cells cultured in 1% FCS conditions grew much more slowly and reached only 7.2×10^4 cells/well at the end of the same period (figure 3.1).

ii) PEO14 cells

PEO14 cells were also seeded in 24 well plates at a concentration of 2.5×10^4 cells/well and cultured in either 1% or 10% FCS conditions. At the end of the 11 days incubation, cells had reached 23×10^4 cells/well while cells in 1% FCS reached 7.5×10^4 cells/well (figure 3.2). Comparing the growth of the two ovarian cancer cell lines, it can be seen that PEO4 cells grow faster than PEO14 cells in 10% serum, and that both cell lines grow much slower but at comparable rates in 1% serum.



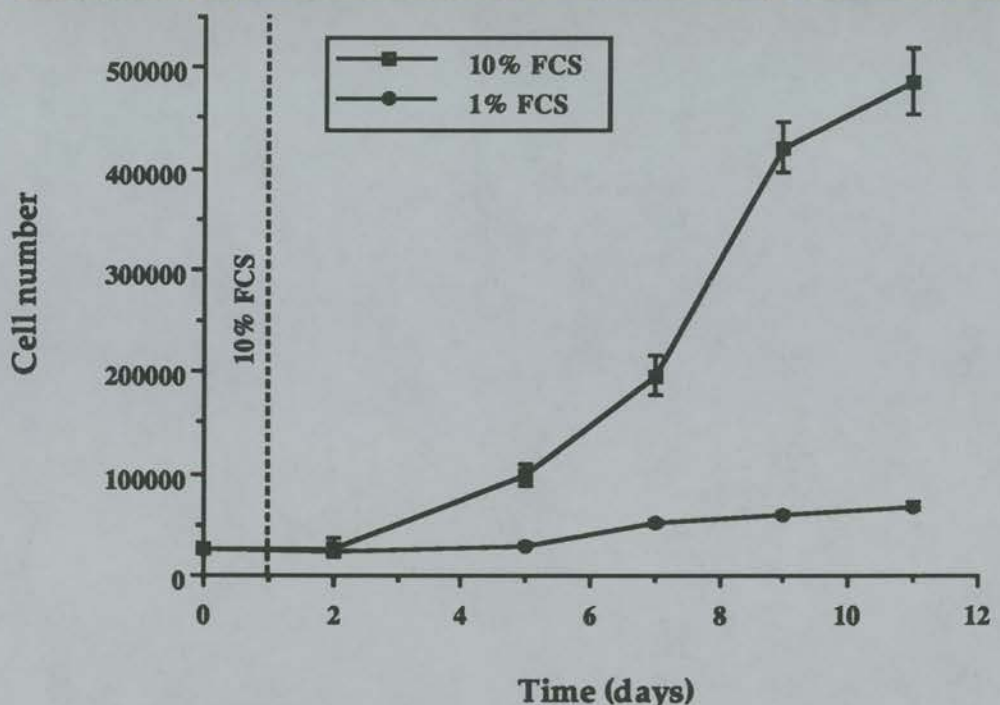
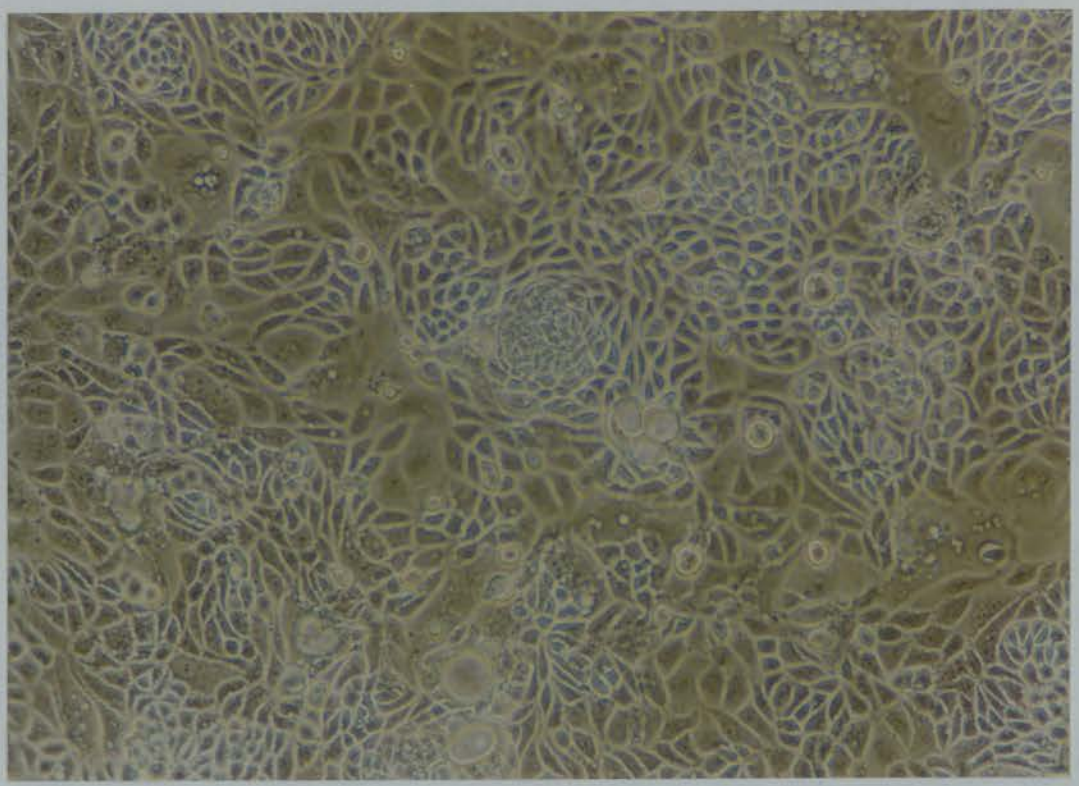


Figure 3.1: Growth of PEO4 ovarian epithelial cancer cells (shown on top) in culture media containing 10% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of 2.5×10^4 cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 10% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.



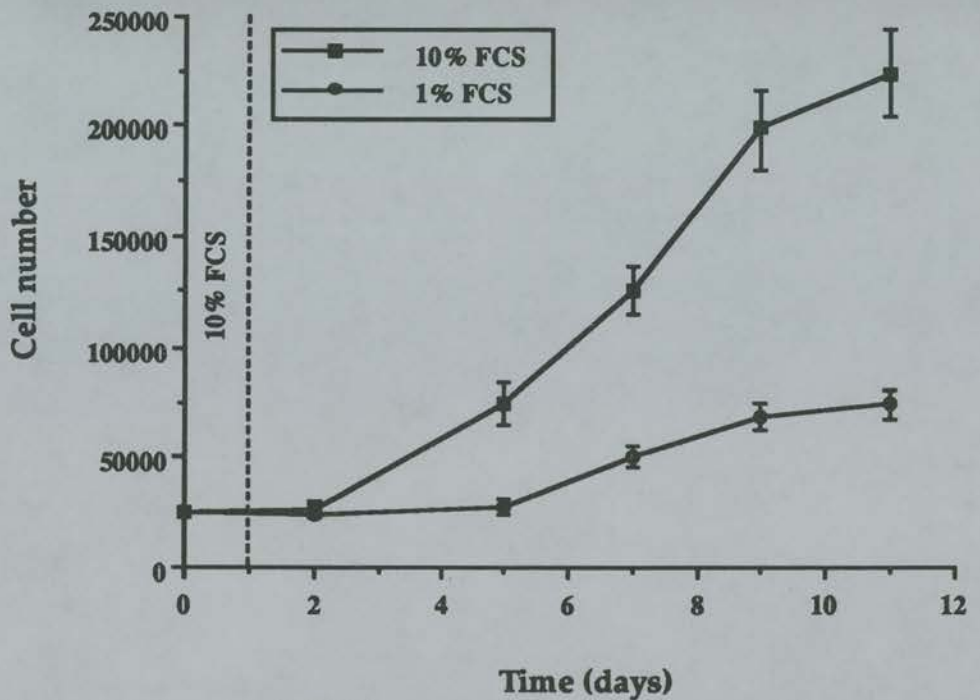


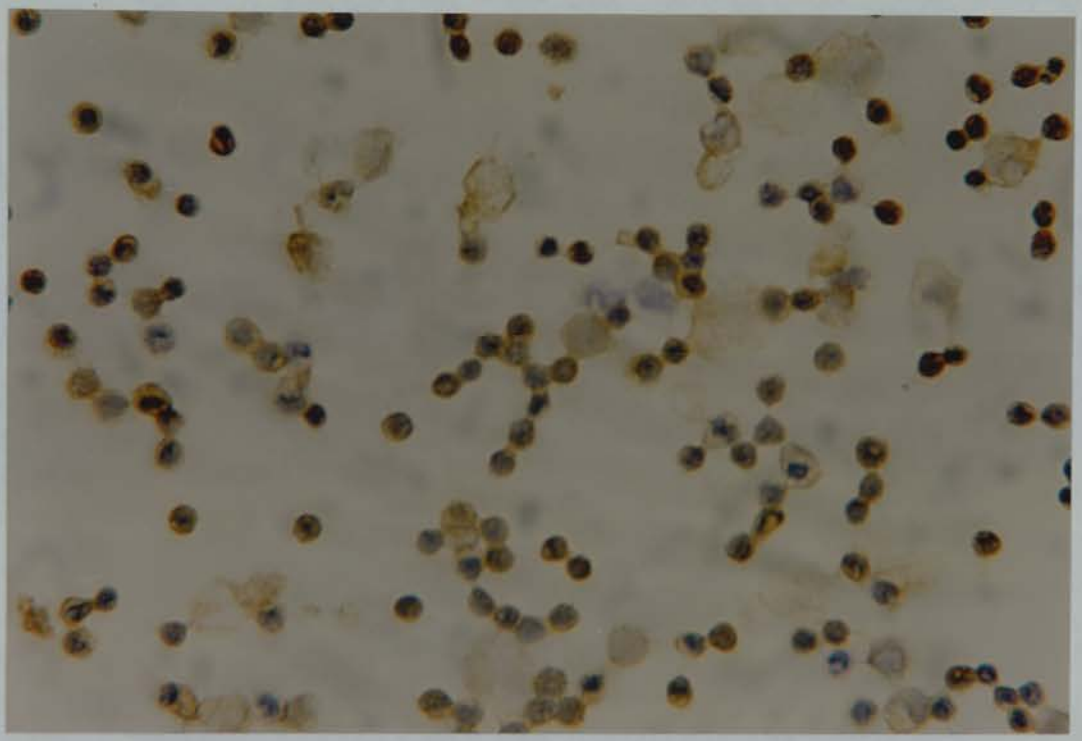
Figure 3.2: Growth of PEO14 ovarian epithelial cancer cells (shown on top) in culture media containing 10% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 10% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.

3.1.2 Ovarian fibroblast cell lines

3.1.2.1 Characterisation

The fibroblasts that were used in the experiments presented in this thesis were derived from ascites of patients with ovarian cancer. The PEO9F and PEO14F cells were derived from patients with adenocarcinoma while PEO12F and PEO27F cells were derived from patients with serous cystadenocarcinoma. Samples of ascites were recovered from liquid nitrogen and seeded into tissue culture flasks. After 6 h, media (15% FCS) were replaced. The fibroblasts which attach more rapidly to the tissue culture plastics were thus selected and once the cultures were established, multispot slides were prepared for immunohistochemistry in order to compare the original ascites with the selected fibroblast culture. The antibodies selected were: leucocyte common antigen, epithelial membrane antigen, fibroblast antigen and ET-1 antibody (not used with ascites). A number of dilutions were used in order to determine the optimum antibody concentrations and controls (no primary antibody addition) were included in each set of multispots. The ascites cell population for the PEO12F cell line consisted predominantly of leucocytes (85%) and contained few fibroblasts (10%) and less epithelial cells (5%). Multispots of the cultured cells stained with the same antibodies showed that almost 99% of the cells were stained with the fibroblast antibody, and very low staining with common leucocyte antigen , ET-1 and epithelial membrane antigen, suggesting that the cultured cells were fibroblasts. The other two cell lines (PEO14F and PEO27F) showed similar results. Examples of the observed staining in the ascites and fibroblasts are shown in figures 3.3, 3.4, 3.5 and 3.6.

Ascites



Fibroblast culture

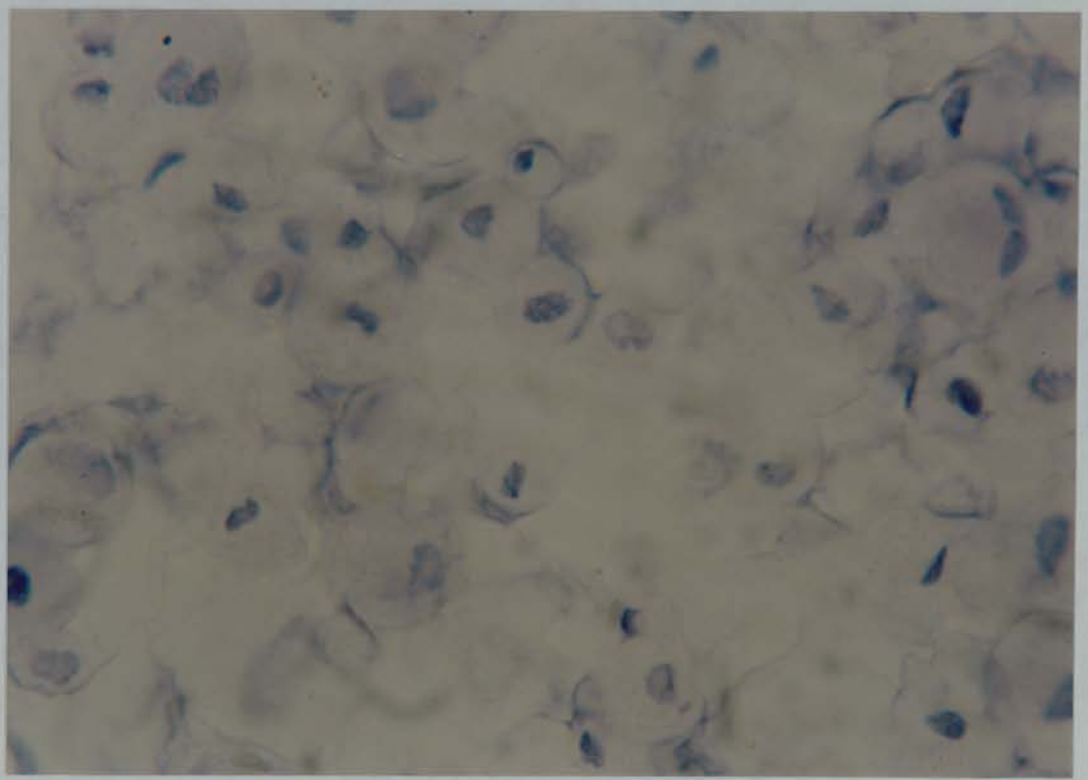
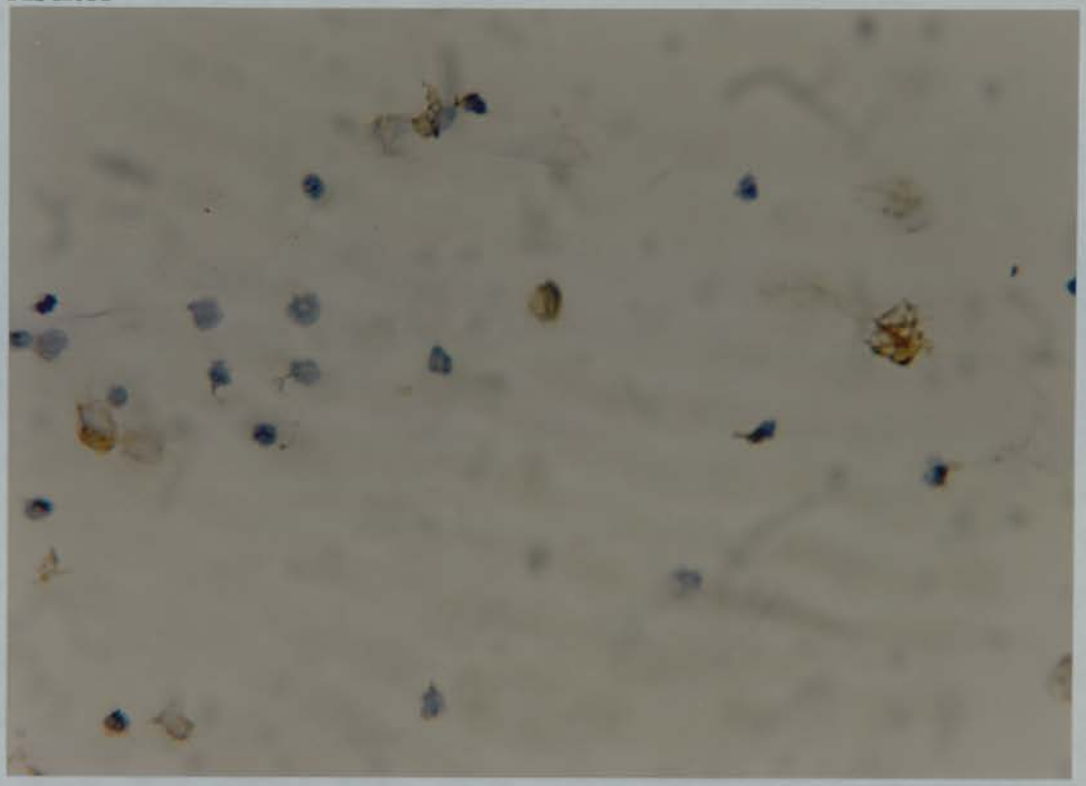


Figure 3.3: Multispot slides stained with leucocyte common antibody

Ascites



Fibroblast culture

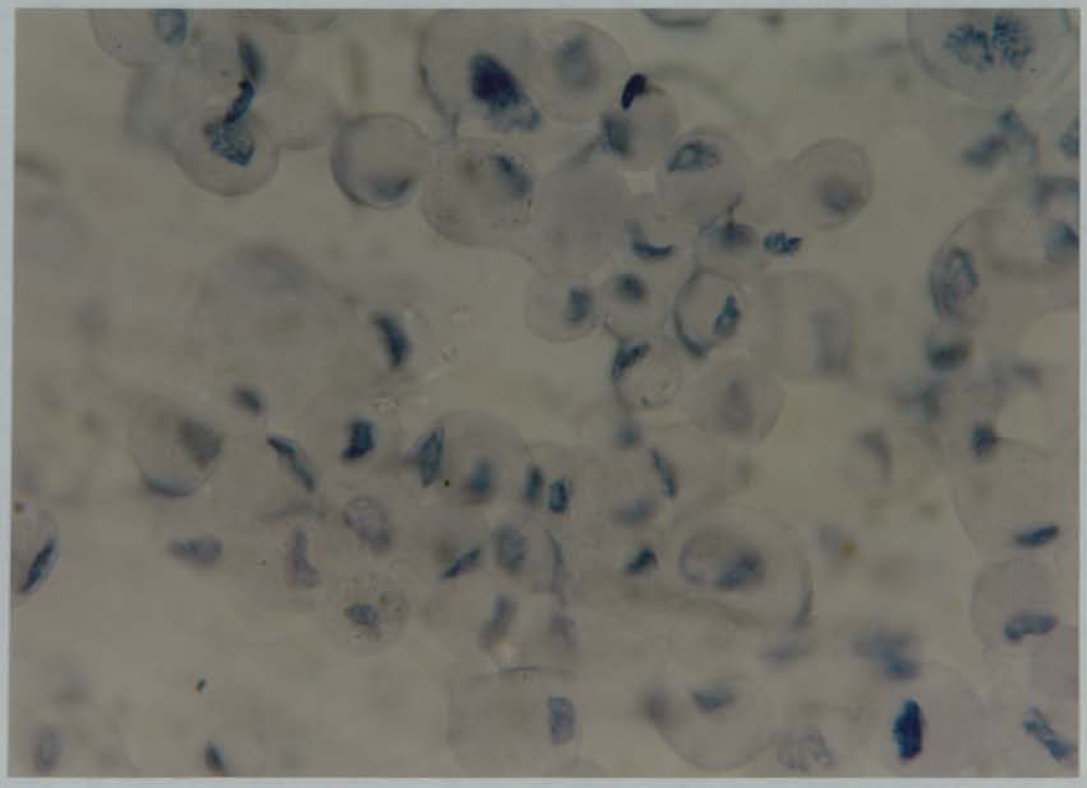
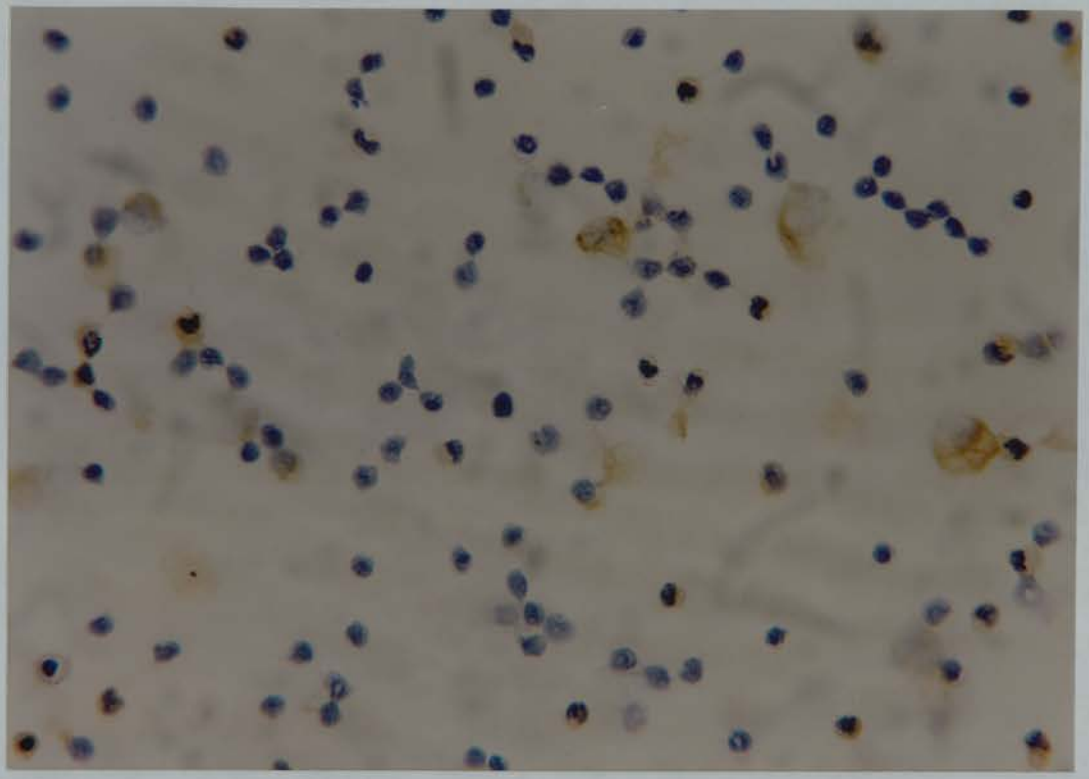


Figure 3.4: Multispot slides stained with epithelial membrane antigen.

Ascites



Fibroblast culture

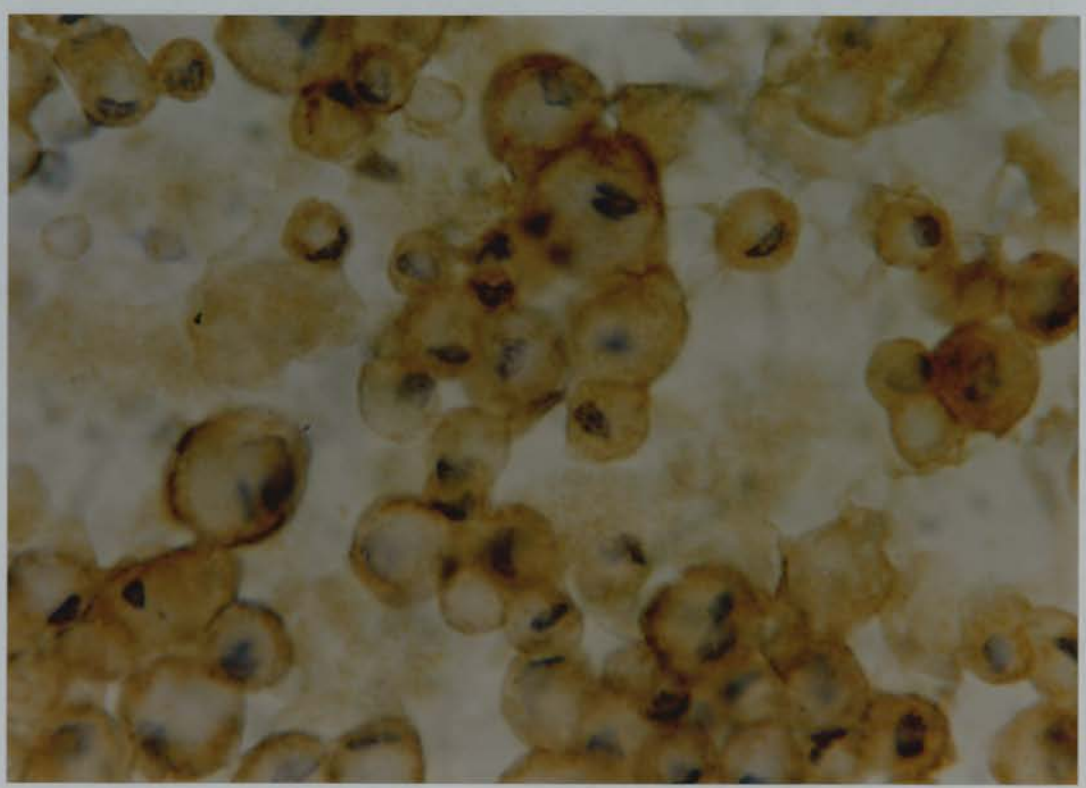


Figure 3.5: Multispot slides stained with fibroblast antigen.

Fibroblast culture

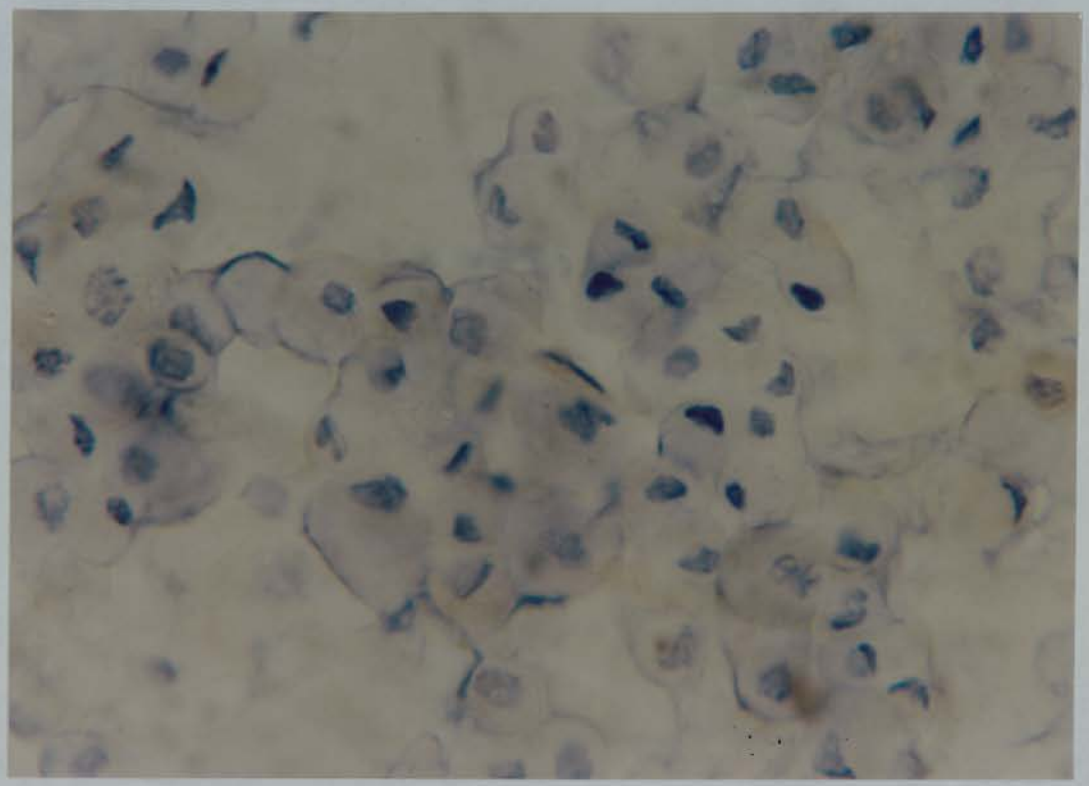


Figure 3.6: Multispot slides stained with ET-1 antibody.

3.1.2.2 Growth

Several culture conditions using a variety of serum concentrations were compared in order to decide optional conditions for the growth of these cells. It was finally decided that ovarian fibroblast cell lines were growing best when cultured in media supplemented with 15% FCS and images of these cell lines are shown in photographs 3.7, 3.8 and 3.9 PEO12F, PEO14F and PEO27F cell lines appear to have a limited period of growth in cell culture after which their rate of growth slows down and they progressively become enlarged and die. In all the experiments performed therefore, cells were used within the first three weeks of their growth.

i) PEO12F

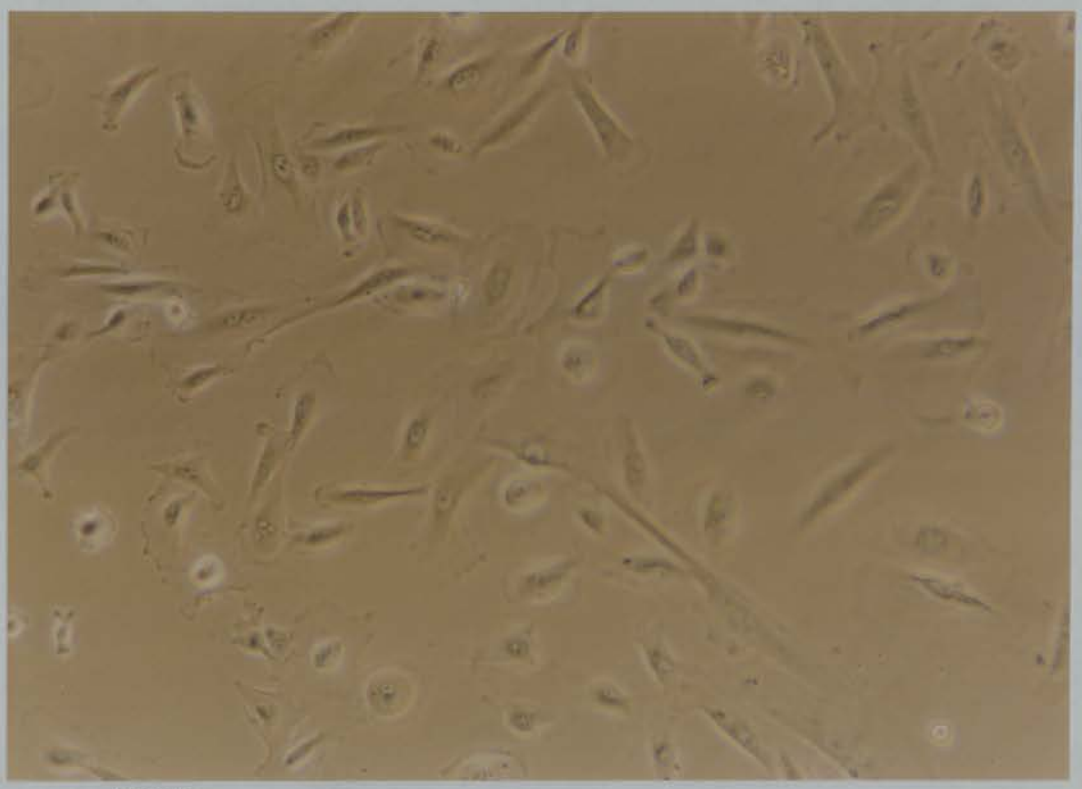
PEO12F fibroblasts were seeded at a concentration of $2.5x10^4$ cells/well in 24 well plates and cultured in 1% and 15% serum for 11 days. Cells growing in 15% serum reached levels of $12x10^4$ cells/well while cells cultured in 1% serum had a far lower rate of growth reaching $5.9x10^4$ cells/well (figure 3.7).

ii) PEO14F

As with PEO12F cells, PEO14F fibroblasts were seeded at a concentration of $2.5x10^4$ cells/well. At the end of the 11 day incubation in 15% serum numbers of cells had reached $11.7x10^4$ cells/well compared to $5.8x10^4$ cells/well for cells growing in 1% serum for the same period of time (figure 3.8).

iii) PEO27F

PEO27F cells seeded at the same concentration as PEO12F and PEO14F fibroblasts also exhibited higher growth rates when cultured in 15% serum, reaching $12.7x10^4$ cells/well compared to $5.9x10^4$ cells/well when cultured in 1% serum (figure 3.9).



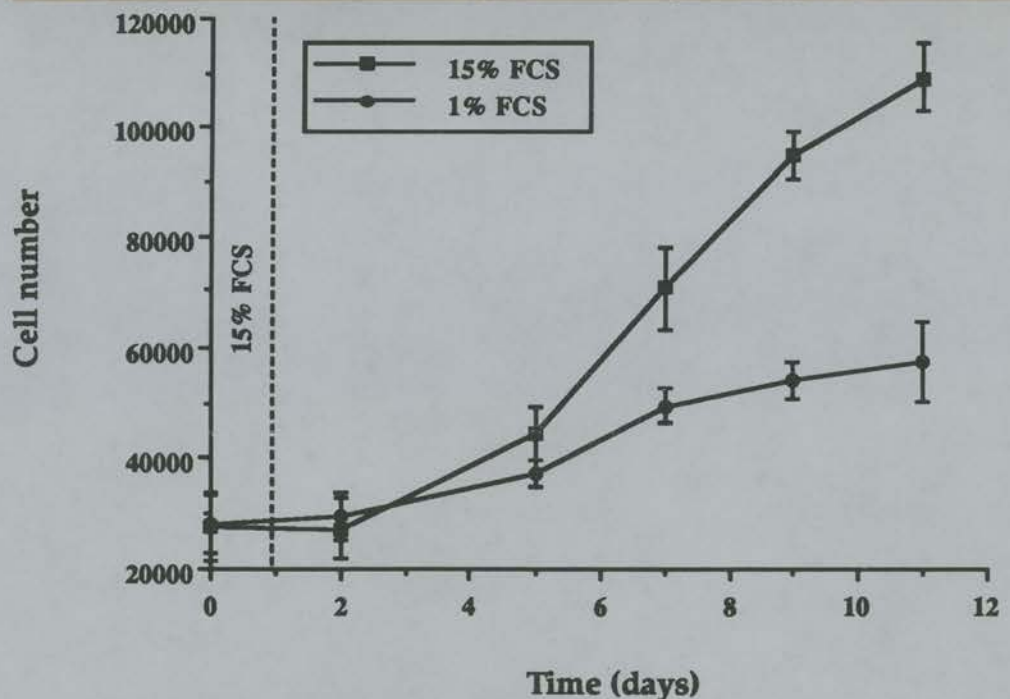
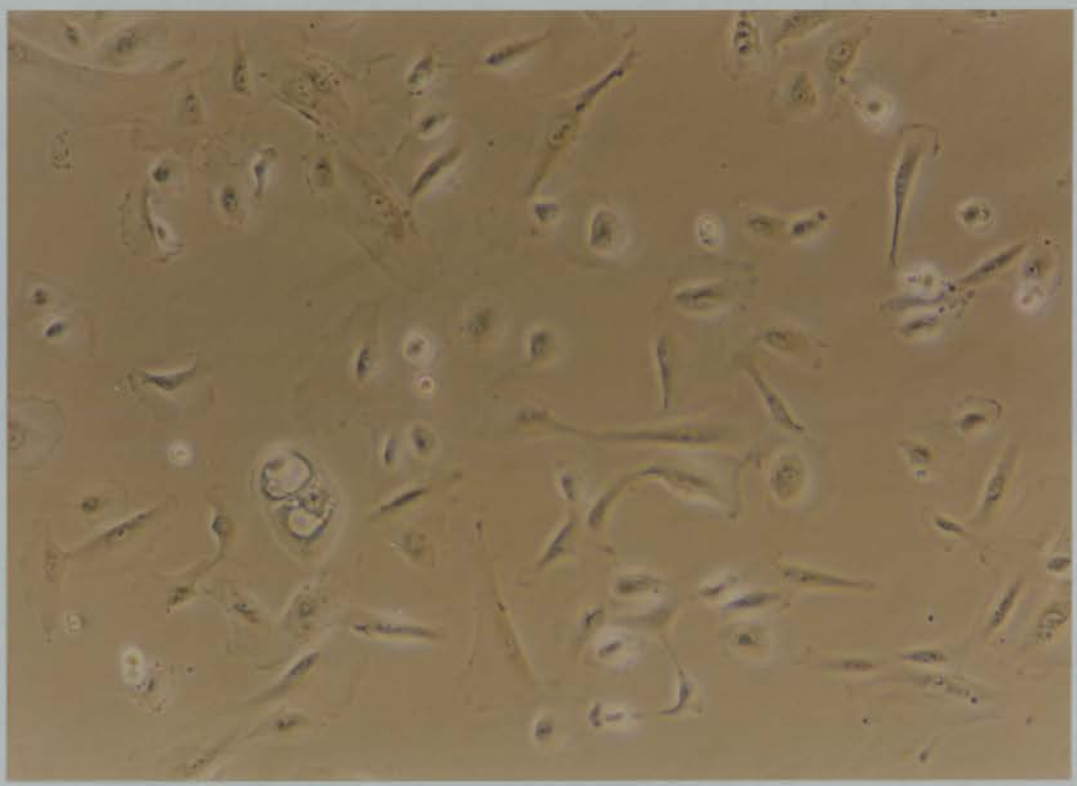


Figure 3.7: Growth of PEO12F ovarian fibroblasts (shown on top) in culture media containing 15% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 15% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.



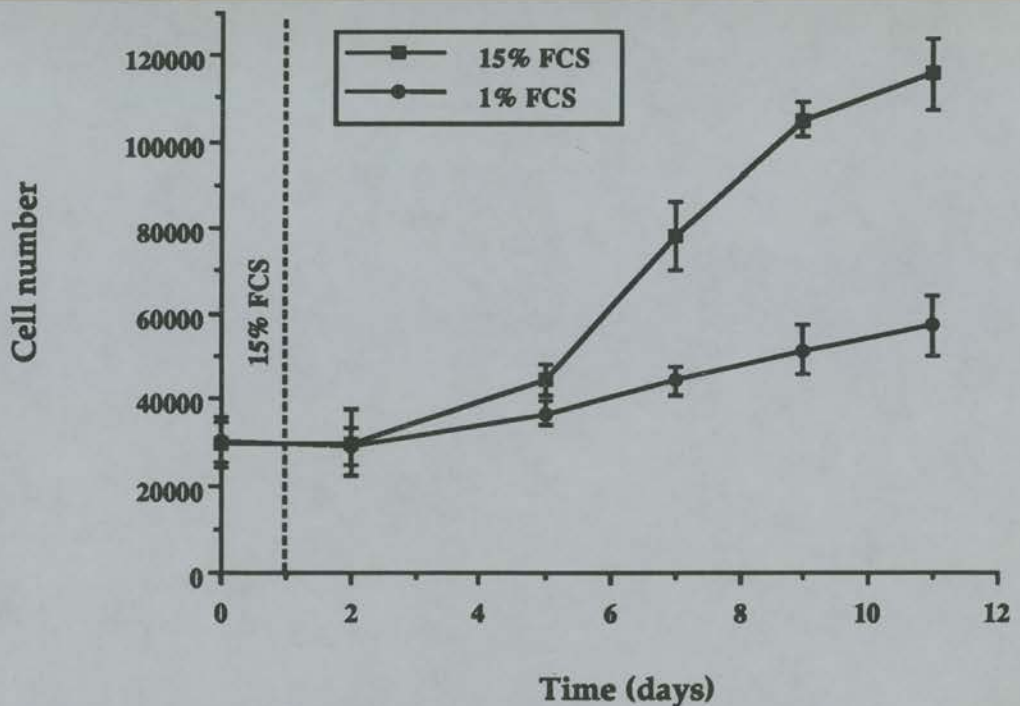
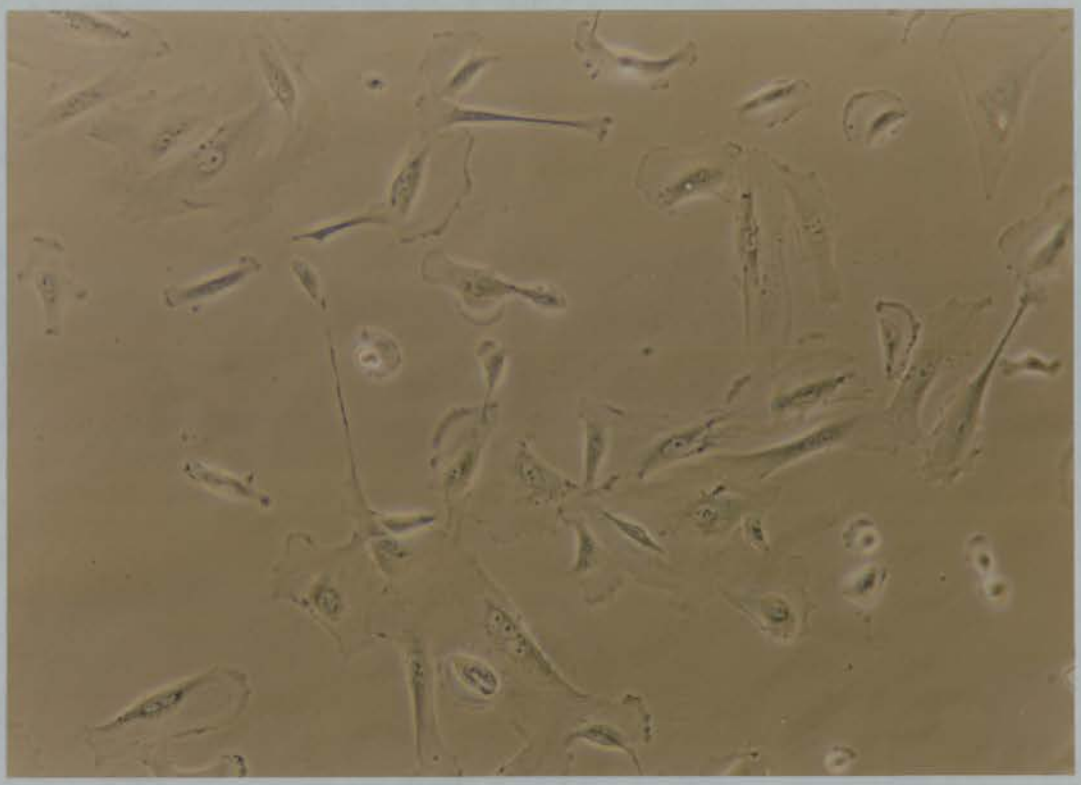


Figure 3.8: Growth of PEO14F ovarian fibroblasts (shown on top) in culture media containing 15% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 15% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.



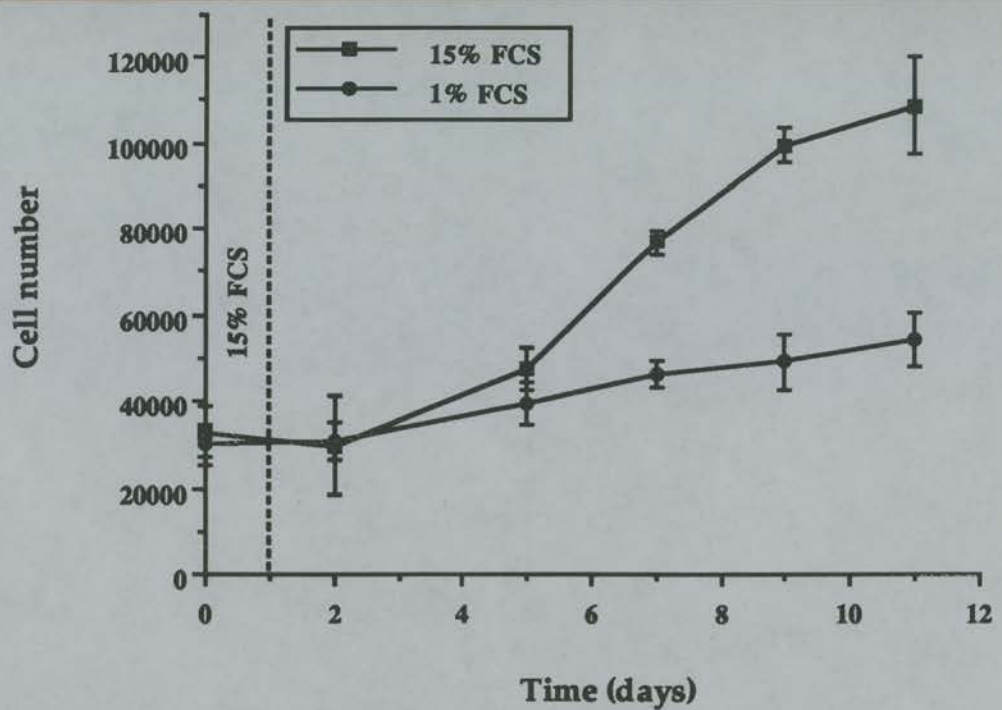


Figure 3.9: Growth of PEO27F ovarian fibroblasts (shown on top) in culture media containing 15% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 15% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.

3.2 Breast cell lines

3.2.1 Breast epithelial cancer cell lines

The three breast carcinoma cell lines MDA-MB-231, T47D and ZR-75-1 were routinely cultured in DMEM culture medium with 10% serum. Photographs and growth curves of the three cell lines are seen in figures 3.10 and 3.11 and 3.12.

i) MDA-MB-231

Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well and cultured in 10% and 1% serum for 11 days. The rate of growth in 10% serum was higher than in 1% serum and growth reached $55x10^4$ cells/well compared to $13x10^4$ cell/well (figure 3.10).

ii) T47D

As with MDA-MB-231 cells, T47D breast cancer cells showed better growth in 10% serum conditions reaching $43x10^4$ cells/well compared to $10x10^4$ cells/well when cultured in 1% serum conditions. Comparing the growth rates of T47D cells with those of MDA-MB-231, it appeared that T47D cells grow more slowly than MDA-MB-231 cells in both 10% and 1% serum conditions (figure 3.11).

iii) ZR-75-1

ZR-75-1 cells were the slowest growing epithelial breast cancer cells of the three cell lines used in the study. However, the same characteristics of growth were observed with ZR-75-1 cells, with 10% serum conditions allowing cells to grow to $16x10^4$ cells compared to $6x10^4$ cells/well in 1% serum conditions (figure 3.12).

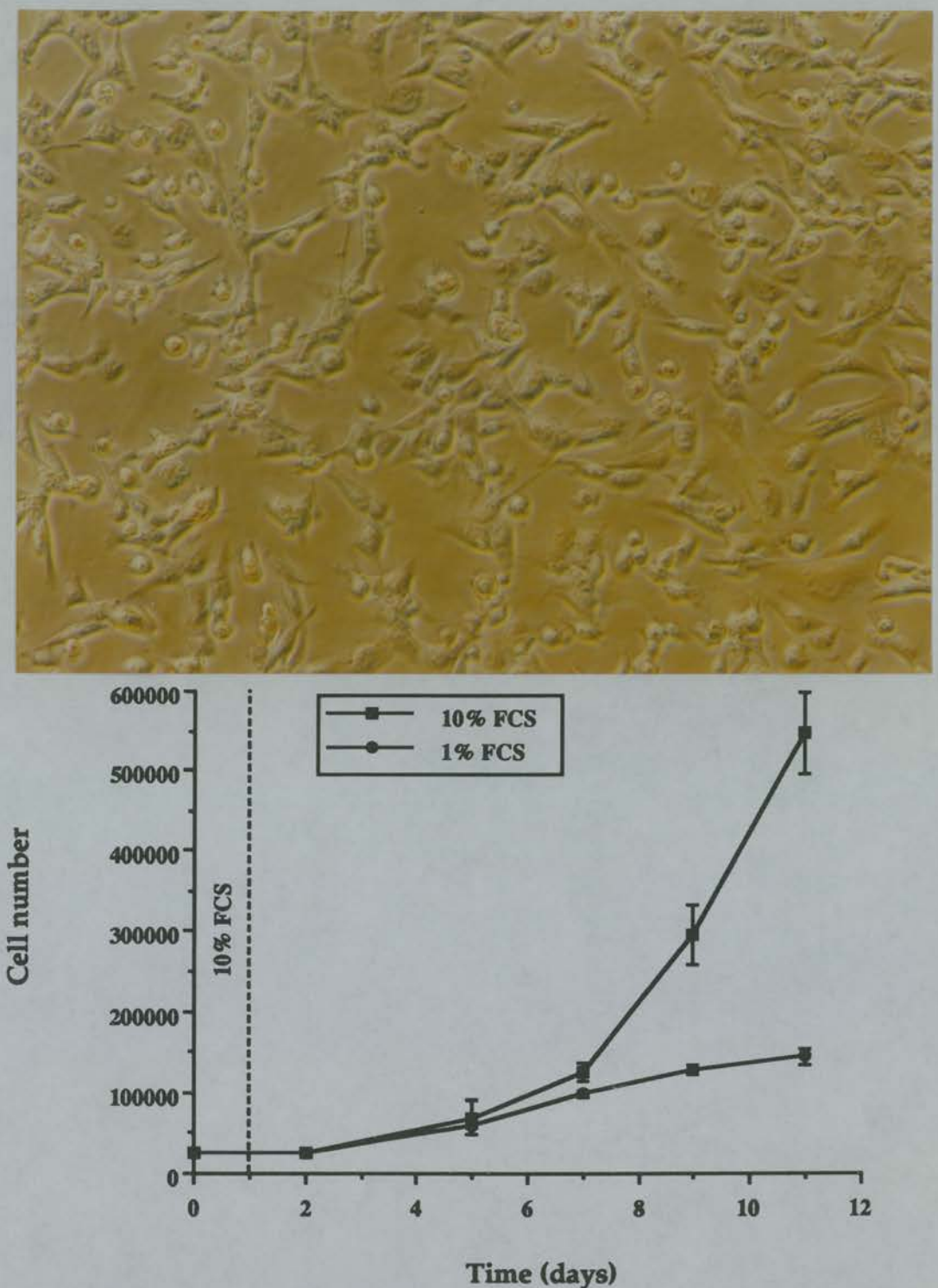


Figure 3.10: Growth of MDA-MB-231 breast epithelial cancer cells (shown on top) in culture media containing 10% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of 2.5×10^4 cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 10% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.

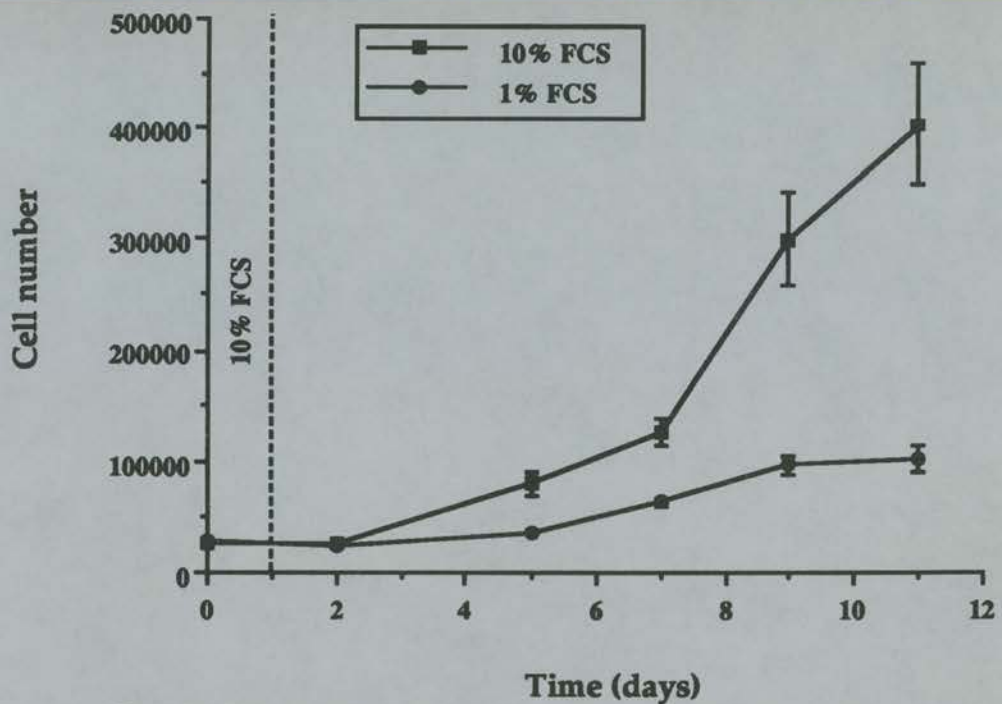
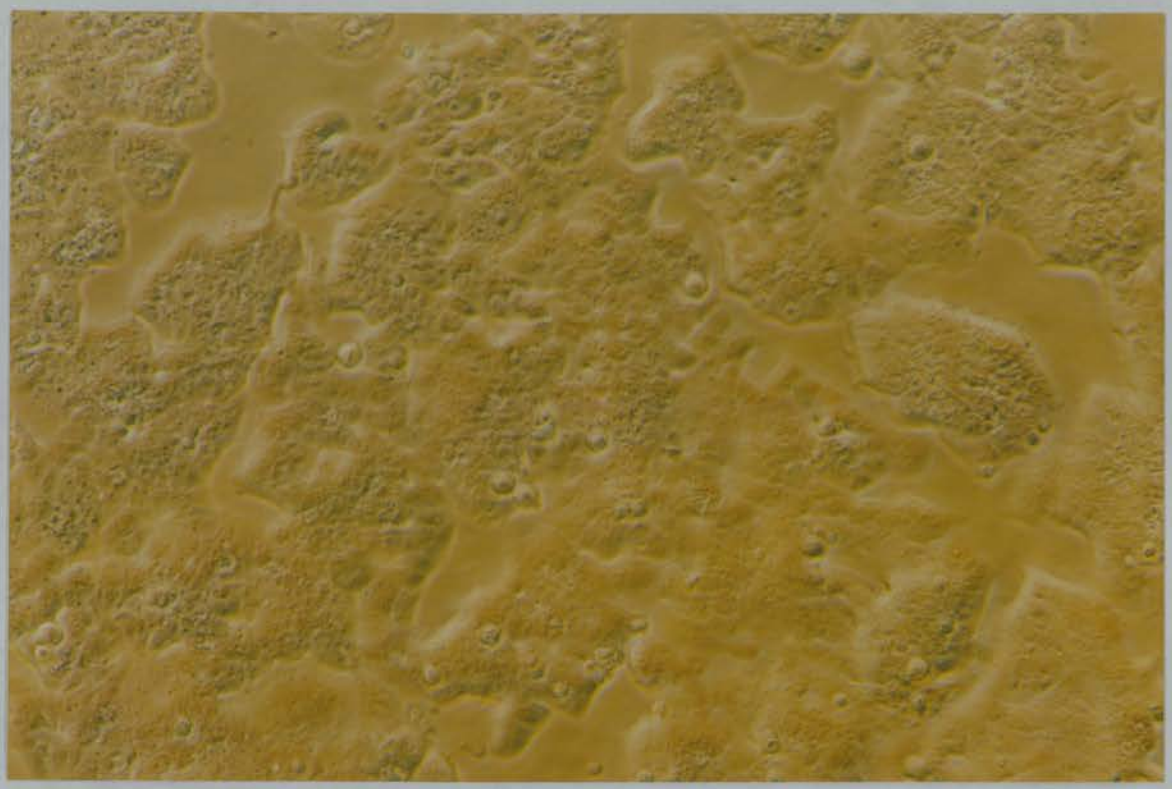


Figure 3.11: Growth of T47D breast epithelial cancer cells (shown on top) in culture media containing 10% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of 2.5×10^4 cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 10% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.



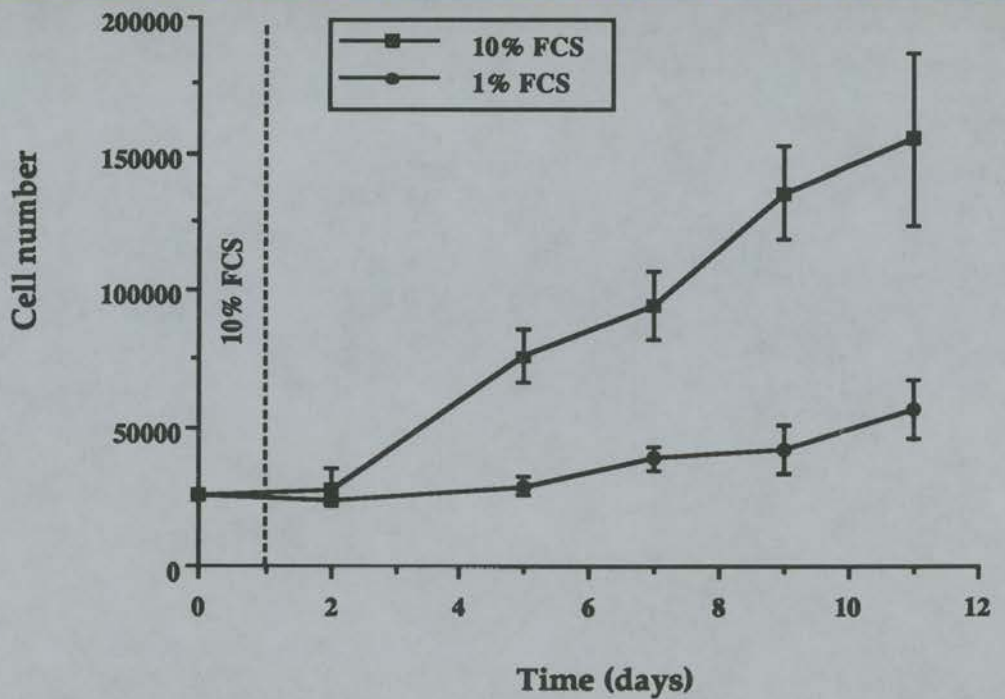


Figure 3.12: Growth of ZR-75-1 breast epithelial cancer cells (shown on top) in culture media containing 10% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 10% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.

3.2.2 Breast fibroblast cell lines

The two breast fibroblast cell lines BRF1 and BRF2 were obtained by Peter Mullen (ICRF Medical Oncology Unit, Edinburgh, personal communication) and previously routinely cultured in Alpha MEM culture medium supplemented with 15%. For these growth experiments, cells were seeded in 24 well plates at $2.5x10^4$ cells/well in 15% serum conditions. Cells were supplemented with fresh medium 24h after seeding and allowed to grow in the same conditions or in 1% serum conditions for 10 days. Photographs of the cell lines and growth curves for the 11 day incubation period under both sets of conditions are shown in figures 3.13 and 3.14.

i) BRF1

As shown in figure 3.13, BRF1 cells exhibited satisfactory growth under both sets of conditions. However, in 15% serum, cells grew faster reaching a cell number of $62x10^4$ cells/well compared to $38x10^4$ cells/well when cultured in 1% serum for the same incubation period (figure 3.13).

ii) BRF2

The growth of the BRF2 cells was very similar to that of BRF-1 fibroblasts. In media supplemented with 15% serum cell number reached $63x10^4$ cells/well and in 1% serum conditions cell number reached $35x10^4$ cells/well (figure 3.14).

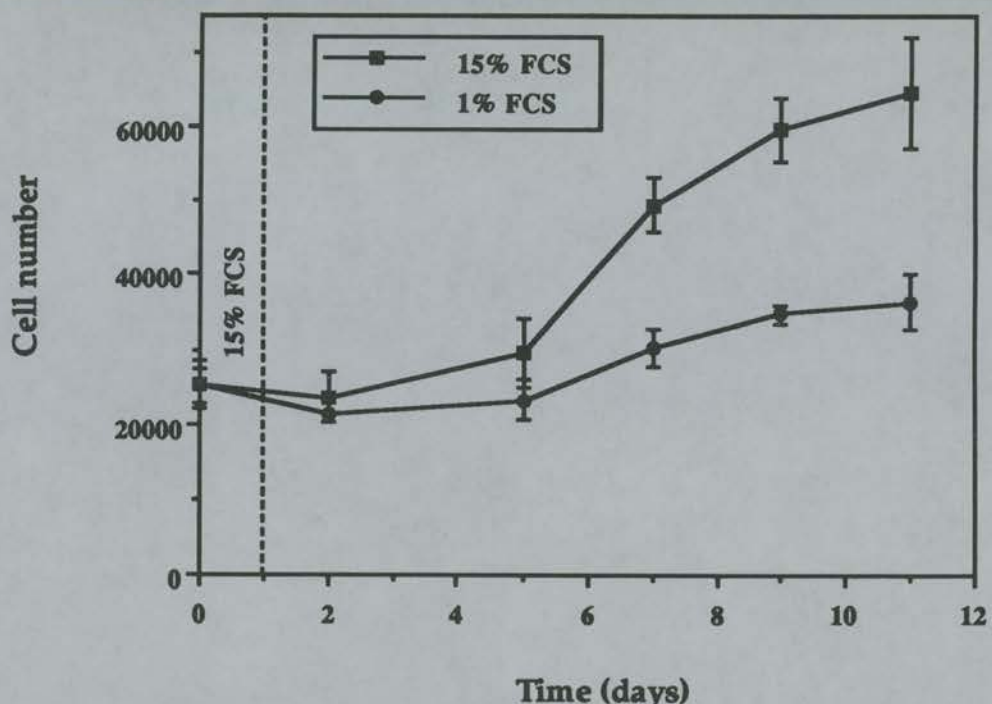
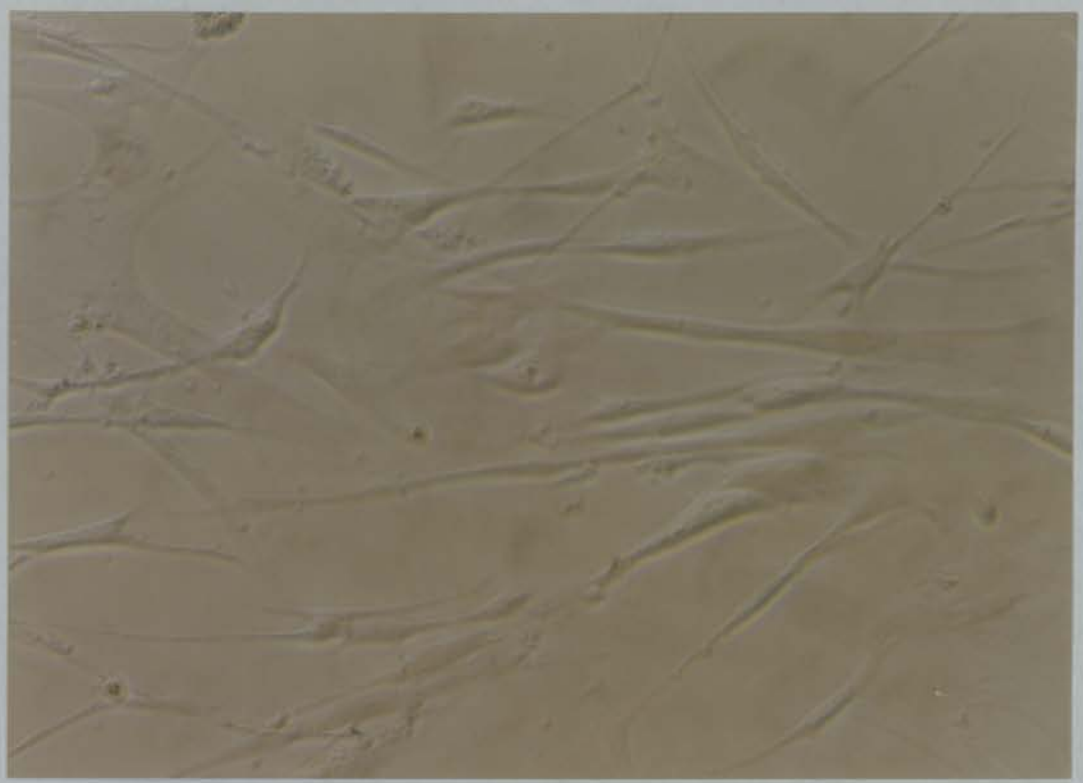


Figure 3.13: Growth of BRF1 breast fibroblasts (shown on top) in culture media containing 15% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 15% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.



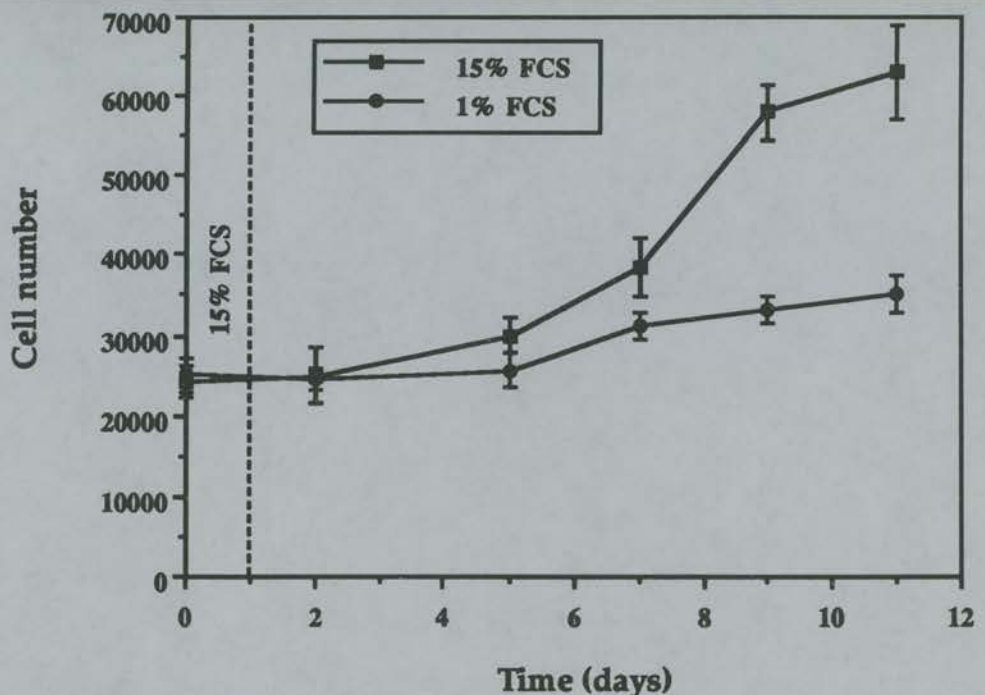


Figure 3.14: Growth of BRF2 breast fibroblasts (shown on top) in culture media containing 15% and 1% foetal calf serum (FCS) during an 11 day incubation period. Cells were seeded in 24 well plates at a concentration of $2.5x10^4$ cells/well in 15% serum conditions. After 24h media fresh media supplemented with either 15% or 1% serum were added in the cultures and cells were incubated for 10 days. Cells counts were obtained on days 0. 2, 5, 7, 9 and 11. Media were replenished once on day 5. Results represent mean±se of quadruplicate values obtained from a representative of 3 experiments performed on separate occasions.

3.4. Discussion

Results presented in this chapter, show the growth characteristics of a number of cell lines that were used as models in order to investigate the role of endothelins in ovarian and breast cancer. A number of experiments using the cell lines would need to be carried out at restricted serum conditions and thus the growth of the cell lines was investigated in full and restricted serum conditions. All the cell lines demonstrated more rapid growth in 10-15% serum. However, even in 1%FCS all the cell lines exhibited some growth during the 11 day incubation period and indicated that these culture conditions can be used in experiments. Additional results show the characterisation of the PEO12F, PEO14F and PEO27F cell lines which were shown to consist of more than 99% fibroblasts.

4.1 Secretion of endothelin by ovarian and breast carcinoma and fibroblast cells

The following section describes the results of experiments designed to assess if ovarian and breast carcinoma and fibroblastic cell lines secrete ET-1-like peptide. The method used to determine such secretion is based on an assay of conditioned media using a radioimmunoassay employing an ET-1 specific primary antibody included in a commercially available ET-1 detection radioimmunoassay kit (Peninsula).

4.1.1 Optimisation of extraction procedure

For the detection of ET-1-like material secreted by different cell lines, conditioned media were collected and concentrated by extracting the material through cartridge columns. Conditioned media (5ml) from two ovarian (PEO4, PEO14) and three human breast epithelial cancer cell lines (MDA-MB-231, T47D, ZR-75-1), three ovarian (PEO12F, PEO14F, PEO27F) and two breast fibroblast cell lines (BRF2, BRF1) as well as mouse S3T3 fibroblasts growing under serum-free conditions, were collected. After collecting the conditioned media, the cells in each flask were trypsinised off the plastic and counted using a coulter counter. Collected conditioned media were extracted through C18 cartridge columns and concentrated to one tenth of their original volume by centrifugation under vacuum and reconstitution in radioimmunoassay (RIA) buffer (500µl).

In order to determine the efficiency of the extraction method, duplicate samples of diluted ^{125}I-ET-1 (1975, 1875 and 1934, 1963 cpm respectively) in serum-free media were loaded onto a C-18 cartridge column and after 60 min at room temperature the bound material was eluted with either 60% acetonitrile or 60% acetonitrile plus 0.1% tri-fluoro-acetic acid. At the end of the extraction, 80% (mean value of two samples: 1597cpm) of radiolabelled ET-1 eluted with 60% acetonitrile was recovered, while 96% (mean value of two samples: 1897cpm) of ^{125}I-ET-1 using 60% acetonitrile,

0.1% tri-fluoro-acetic acid, was collected. Since the latter extraction proved more efficient, the extractions of the conditioned media collected from all the cell lines in culture were performed using 60% acetonitrile, 0.1% tri-fluoro-acetic acid for the elution of the material bound on the C-18 cartridges.

4.1.2 Radioimmunoassay optimisation and specificity

Before the radioimmunoassay was routinely used to detect the level of peptide secreted by different cell lines, several control experiments were performed in order to optimise the method and determine its specificity. The data sheet supplied with the radioimmunoassay kit suggested low cross-reactivity of the ET-1 peptide with the isopeptides ET-2 and ET-3 (7%). To confirm these levels of cross-reactivity, serial dilutions of ET-2 and ET-3 (128, 64, 32, 16 and 8 pg), and ET-1 (128, 64, 16, 4 and 1 pg), were run through the assay and were compared to the serial dilutions of the standard ET-1 included in the RIA kit (figure 4.1).

Figure 4.1 shows that ET-1, ET-2 and ET-3 compete with the radiolebelled ET-1 in the radioimmunoassay. However, ET-1 dilutes in an almost identical way with the standard peptide while the addition of ET-2 and ET-3 give somewhat different results. The cross-reactivities of ET-2 and ET-3 with the assay's standard ET-1 were estimated to be 14.4% and 8.3% respectively, while ET-1 that had been obtained from different sources behaved identically with the standard ET-1 included in the kit. Two completely unrelated peptides [bombesin and interleukin-6 (IL-6)] were also run through the assay and results suggested that no cross-reactivity of standard ET-1 was detected with either bombesin or IL-6. Table 4.1 shows the amounts of ET-1, ET-2 and ET-3 added in diluted samples of the peptides in the assay in order to determine the level of cross-reactivity. The results of immunoreactive ET-1-like material

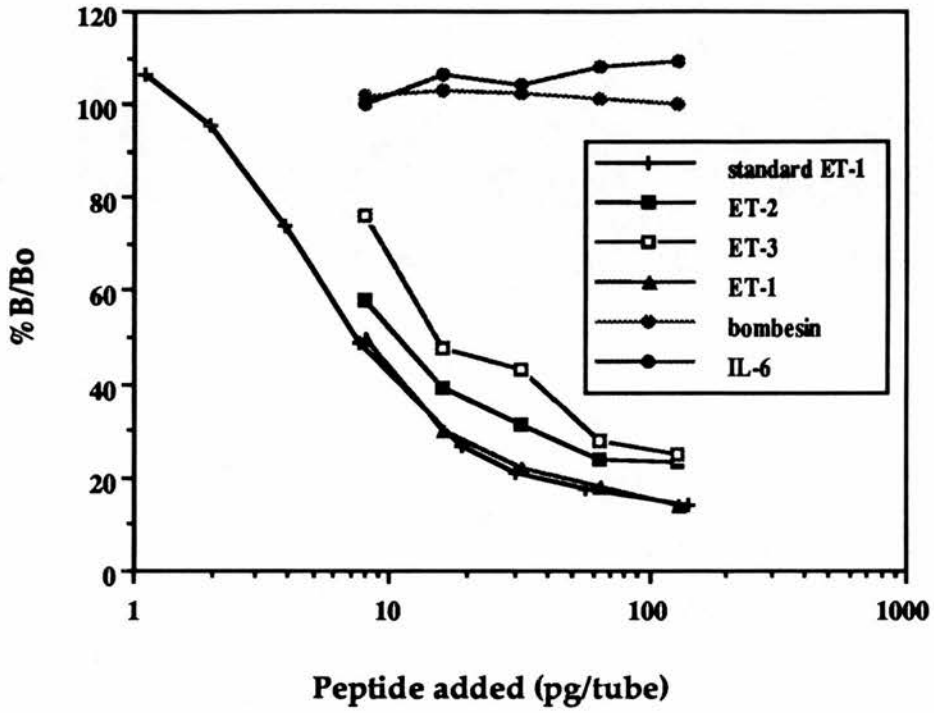


Figure 4.1: Graph shows the parallel dilution of standard ET-1 included in a ^{125}I-ET-1 radioimmunoassay kit with ET-1, ET-2, ET-3 obtained separately. Dilutions of two unrelated peptides bombesin, and interleukin-6 showed little displacement and no parallelism with standard ET-1. Graph is a typical example of 3 experiments performed on separate occasions.

	ET-1					ET-2					ET-3				
ET added (pg)	1	4	16	64	128	8	16	32	64	128	8	16	32	64	128
ET-1 detected (pg)	0.96	3.6	15.7	61.9	118.1	1.2	2.2	5.3	8.6	16.9	0.2	2.0	3.3	5.2	10
% cross reactivity	96	90	98.1	96.7	92.3	15	13.7	16.6	13.4	13.2	2.5	12.5	10.3	8.1	7.8
Mean % cross reactivity	94.7±3					14.4±1					8.24±2				

Table: 4.1: Table of the cross reactivity of standard ET-1 included in the radioimmunoassay kit with ET-1, ET-2 and ET-3 which had been obtained separately. First row [ET added (pg)] represents the amount of ET added in pg in the assay, the second row [ET-1 detected (pg)] represents the amount of ET-1-like material detected in each sample after it has been run through the assay and row 3 represents the % cross-reactivity of each of the samples with standard ET-1. The mean %cross-reactivity values±sd for each ET with standard ET-1 are summarised in row 4. Results represent a typical of 3 experiments performed on separate occasions.

detected in each sample and the % of cross-reactivity for each sample and the mean % cross-reactivity for all dilutions are shown. Due to the existence of cross-reactivities between the three ET isopeptides, the material measured by this radioimmunoassay method was referred to as "ET-1-like" material.

Because reports have suggested the presence of ET peptides in serum, all the cell cultures used for the collection of conditioned media were growing in culture media deprived of foetal calf serum and supplemented with essential supplements (HITS, see section). To test the presence of immunoreactive ET-1 in foetal calf serum and the media enrichment (HITS) serial dilutions (neat, 1:1, 1:2, 1:4, 1:8) of the two were assayed. Mean values of measurements obtained on separate occasions suggested significant amounts of ET-1-like material present in serum (0.22pM) while ET-1-like peptide was not detected in HITS supplemented media. The limit of detection for ET-1-like material is <1 pg/tube (40 fM). Finally, in order to test the variability of results due to error of the available technology or handling of the materials, mean intra-assay and inter-assay variations were calculated running an undiluted sample of conditioned media on four separate occasions through the assay. The inter- and intra-assay variations were found to be 5.5 and 6.8% respectively.

4.1.3 Secretion of ET-1-like material by ovarian and breast cell lines

For the detection of ET-1-like material secreted by different cell lines, a neat sample and serial dilutions (1:1, 1:2, 1:4, 1:8) of the collected conditioned media were prepared using RIA buffer.

The samples were assayed and the obtained values for ET-1-like material present in each sample (in pg) were corrected for the dilution factor. Additional corrections were made for the number of cells in the tissue culture flasks from which the conditioned media were derived. Secreted amounts of ET-1-like material were expressed as fmol/10^6 cells/72h.

Table (4.2) summarises the mean values (of quadruplicate experiments performed on separate occasions) of immunoreactive ET-1-like material detected in serial dilution samples of the conditioned media collected from each cell line.

Concentrations of ET-1-like material secreted in the conditioned media by PEO4 and PEO14 ovarian carcinoma cell lines were 1.7±0.42 (mean±sd) and 17.5±7.6 (mean±sd) fmol/10^6 cells/72h respectively. The breast epithelial cancer cell lines MDA-MB-231, T47D and ZR-75-1 cells secreted 14±3.6, 52±12.3 and 17.69±2.56 (mean±sd) fmol/10^6 cells/72h respectively. Although all the epithelial carcinoma cell lines secreted measurable amounts of immunoreactive ET-1-like material, none of the human ovarian and breast or the mouse S3T3 fibroblasts secreted levels of the peptide measurable by this method. All the conditioned media samples collected by the ovarian and breast epithelial cancer cell lines, diluted in parallel with standard ET-1 in the assay as shown in figures 4.2 and 4.3.

The release of ET-1-like material from all the ovarian and breast epithelial cancer cell lines as a function of time over a 48h period was determined by collecting and assaying conditioned media at different time points (6, 12, 24 and 48h). These results are shown in figure 4.4. Although each cell line secreted different amounts of the peptide, the pattern of the release as a function of time was similar for all the cell lines, i.e. seem to increase linearly over the first 12 hours and plateauing thereafter.

Production of immunoreactive ET-1-like material

Cell line	Cell type	pg/tube	pM	cell number (x 10^6)	fmol /10^6 cells
PEO4	human ovarian carcinoma	19.89±4	0.33±0.08	4.7	1.7±0.42
PEO14	human ovarian carcinoma	34.42±1.4	3.5±1.52	0.8	17.5±7.6
PEO12F	human ovarian fibroblasts	<1	-	0.92	-
PEO14F	human ovarian fibroblasts	<1	-	0.96	-
PEO27F	human ovarian fibroblasts	<1	-	0.9	-
MDA-MB-231	breast carcinoma	11.85±3.0	2.8±0.73	3.4	14±3.6
T47D	breast carcinoma	77.94±18.4	10.4±2.46	6.02	52±12.3
ZR-75-1	breast carcinoma	18.28±2.64	3.53±0.51	4.15	17.7±2.56
BRF2	breast fibroblasts	<1	-	0.58	-
BRF1	breast fibroblasts	<1	-	0.64	-
S3T3	mouse fibroblasts	<1	-	0.38	-

Table 4.2: Table on the production of immunoreactive ET-1-like material in conditioned media from ovarian and breast epithelial cancer cell lines and fibroblasts. Results are presented as raw data (pg/tube) or normalised data taking into account the number of cells from which the conditioned media were secreted (fmol/10^6 cells and pM). Results represent mean values±sd of quadruplicate experiments performed on separate occasions.

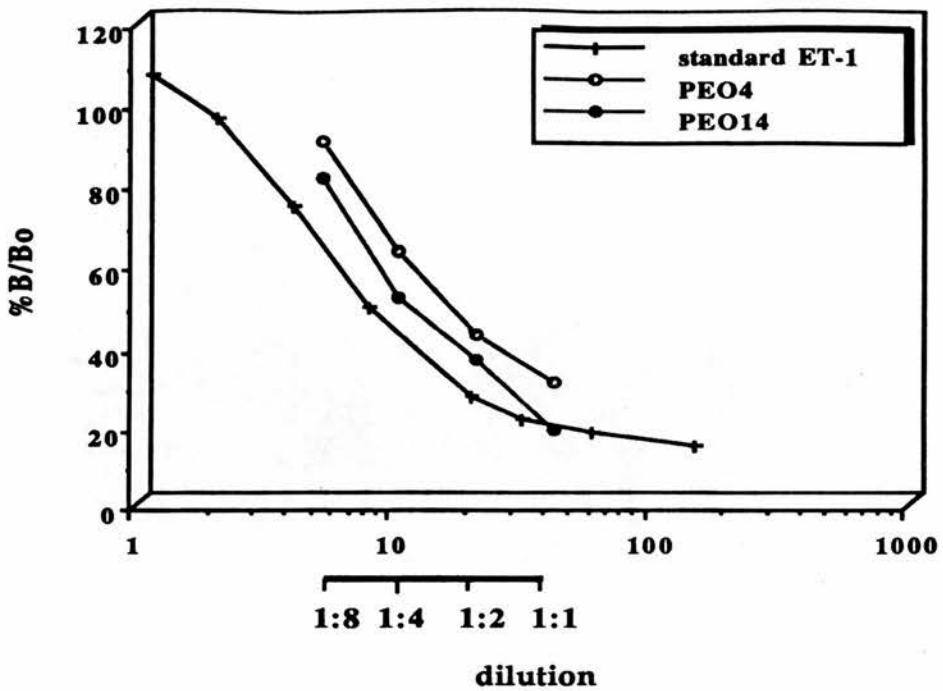


Figure 4.2: Parallel dilution of standard ET-1 with immunoreactive ET-1-like material detected in diluted samples (1:1, 1:2, 1:4, 1:8) of the conditioned media of PEO4 and PEO14 ovarian epithelial cancer cell lines. Graph represents data typical of 3 experiments performed on separate occasions

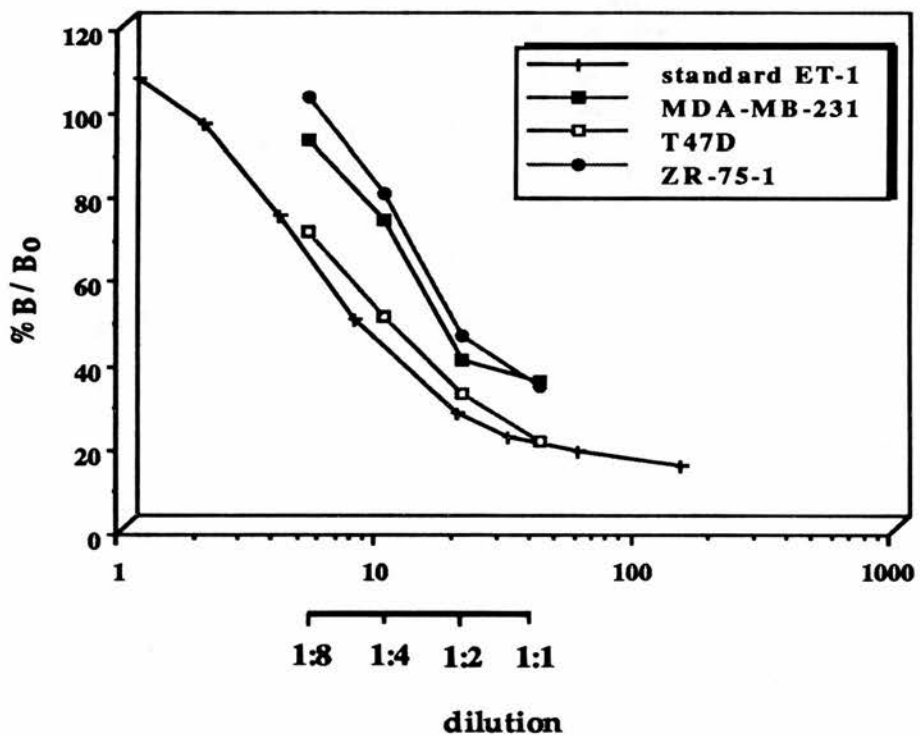


Figure 4.3: Parallel dilution of standard ET-1 with immunoreactive ET-1-like material detected in diluted samples (1:1, 1:2, 1:4, 1:8) of the conditioned media of MDA-MB-231, T47D and ZR-75-1 breast epithelial cancer cell lines. Graph represents data typical of 3 experiments performed on separate occasions.

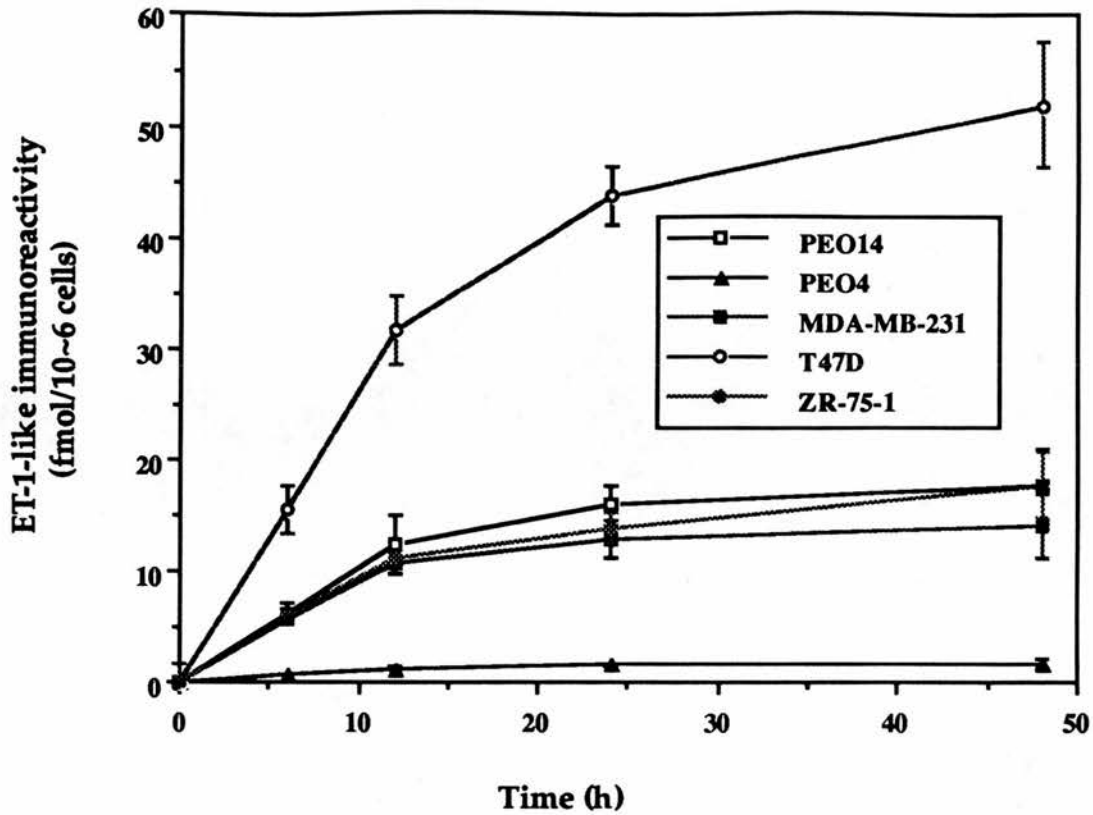


Figure 4.4: Release of immunoreactive ET-1-like material from PEO4 and PEO14 ovarian epithelial and MDA-MB-231, T47D and ZR-75-1 breast epithelial cancer cells growing under serum-free conditions as a function of time. Conditioned media were collected at 6, 12, 24 and 48h. The results represent mean values±sd of 3 distinct conditioned media collected on separate occasions.

4.1.4 Modulation experiments

Endothelin is known to be regulated by several growth factors and cytokines. In human breast cancer factors such as bombesin, insulin and interleukin-6 (IL-6) have been shown to modulate ET-1 expression but no such information is known about human ovarian cancer cell lines. In order to confirm the observed effects in human breast cancer cell lines and to investigate possible modulation effects in human ovarian cancer cell lines, bombesin and IL-6 were added in the serum-free culture media of the cell lines 24 h prior to the collection of the conditioned media. As is shown in figures 4.5 and 4.6 such additions resulted in an increase in basal secretion of ET-1-like material from all the epithelial cell lines. In the ovarian epithelial cancer cell lines, PEO14 cells show the greater increase in ET-1-like secretion with the addition of bombesin (18 pM compared to 4 pM control secretion) while in breast cancer cell lines T47D cells show the greatest increase in secretion of ET-1-like peptide (62 pM compared to 10 pM basal secretion). The percentage increase in ET-1-like peptide secretion from all the cell lines as a result of the addition of bombesin and IL-6 in the culture media is summarised in table 4.3. PEO4 and PEO14 human ovarian epithelial cancer cells showed a 40% and 400% increase (respectively) in basal ET-1-like secretion as a result of the addition of 100 nM bombesin for 24 h and a 38% and 310% increase (respectively) with the addition of 200 ng/ml of IL-6 for the same time period (24 h). Human breast epithelial cancer cell lines (MDA-MD-231, T47D and ZR-75-1) also secreted increased (compared to control) levels of ET-1-like material as the result of the addition of 0.1 μM bombesin (52%, 496% and 57% respectively) or 200 ng/ml IL-6 (26%, 80% and 37% respectively) in the culture media for 24 h. No effects of ET-1-like material secretion were observed with the addition of bombesin or IL-6 in the culture media of human ovarian and breast or mouse S3T3 fibroblasts. The addition of similar amounts of pure bombesin or IL-6 in the RIA had no effect on the detection of immunoreactive ET-1-like peptide (section 4.1.1).

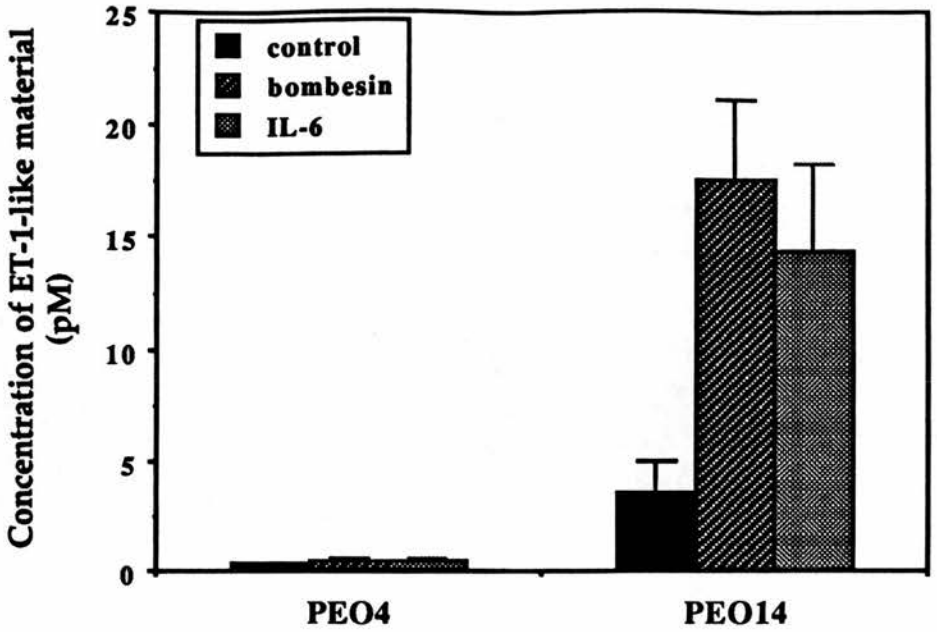


Figure 4.5: Immunoreactive ET-1-like material released from PEO4 and PEO14 cells growing in serum-free media after 72h incubation alone (control) or in serum-free media supplemented with bombesin (100nM) or interleukin-6 (IL-6, 200ng/ml) 24h prior to collection. Bars represent mean values±sd of 3 experiments performed on separate occasions.

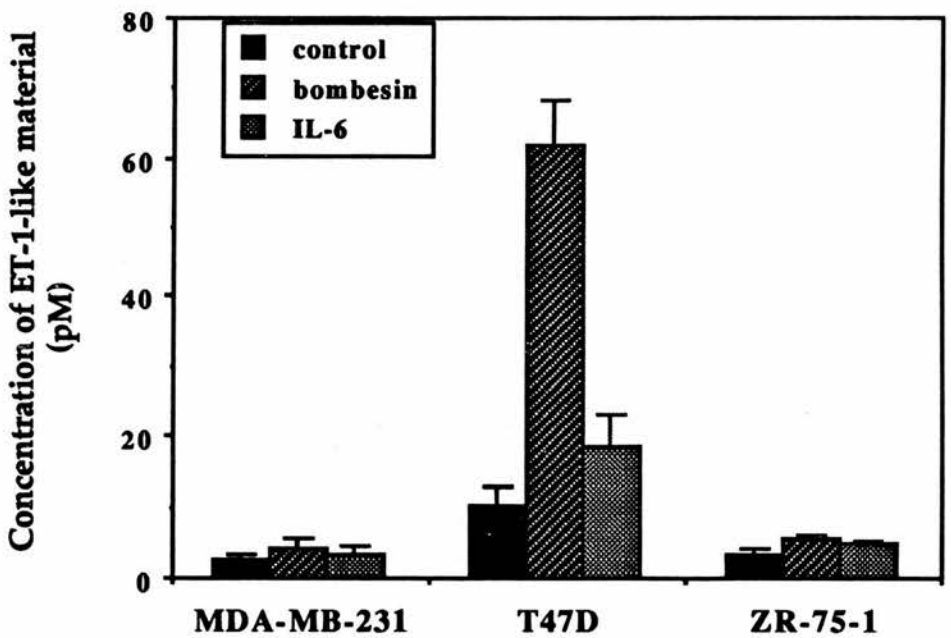


Figure 4.6: Immunoreactive ET-1-like material released from MDA-MB-231, T47D and ZR-75-1 cells growing in serum-free media after 72h incubation alone (control) or in serum-free media supplemented with bombesin (100nM) or interleukin-6 (IL-6, 200ng/ml) 24h prior to collection. Bars represent mean values±sd of 3 experiments performed on separate occasions.

Effect of the addition of bombesin and IL-6 on the secretion of ET-1-like material

Cell line	Cell type	Bombesin (% control)	Interleukin-6 (IL-6) (% control)
PEO4	ovarian carcinoma	140%	138%
PEO14	ovarian carcinoma	500%	410%
MDA-MB-231	breast carcinoma	152%	126%
T47D	breast carcinoma	620%	180%
ZR-75-1	breast carcinoma	157%	137%

Table 4.3: Table of the effects of the incubation of bombesin (100nM) and interleukin-6 (IL-6, 200ng/ml) for 24h in the culture of ovarian and breast epithelial cancer cells, regarding the secretion of immunoreactive ET-1-like material from the cells into conditioned media after a 72h incubation at serum-free conditions. Table represents data from a typical example of 3 experiments performed on separate occasions.

4.2 Discussion:

The results presented above show that ovarian (PEO4 and PEO14) and breast (MDA-MB-231, T47D and ZR-75-1) cancer cell lines secrete immunoreactive ET-1-like material and that levels of ET-1 secreted can be modulated by bombesin and IL-6 peptides. However, as determined by radioimmunoassay of conditioned media, neither ovarian (PEO12F, PEO14F and PEO27F) nor breast fibroblast cell lines (BRF1 and BRF2) produce detectable amounts of the same peptide.

In this study, a commercially available ET-1-detection radioimmunoassay kit was used in order to investigate the production of this peptide from breast and ovarian cancer cell lines and fibroblasts. The specificity of the radioimmunoassay was tested with the use of two unrelated peptides (bombesin and IL-6) which failed to cross react with the assay while ET-1 which had been obtained separately from the kit demonstrated a 94.7% cross reactivity with the standard ET-1 included in the assay. The cross-reactivity of ET-1 with ET-2 and ET-3 isopeptides was also measured and calculated as 14.4 and 8.2% respectively. The assay did not cross-react with "big"-ET-1 as suggested by the manufacturer. These levels of cross reactivity suggest that what has been measured in the assay is mostly ET-1 because for ET-2 and ET-3 crossreactivities to account for these levels it would mean that they are secreted at extremely high levels. However, the possibility that ET-2 and ET-3 could account for a part of the detected amount, could not be ruled out. Previous studies have demonstrated the production of immunoreactive ET-1 in the nervous and cardiovascular systems, pancreatic and colon carcinomas (Kusuhara et al., 1990, Suzuki et al., 1989), pancreatic cancer cells (Oikawa et al., 1994), human breast cancer tissues (Yamashita et al., 1993). In the case of ovarian cancer, only a single report has previously been published and this suggested the production of immunoreactive ET-1 from OVCA-433, A2780 and SKOV-3 ovarian epithelial cancer cell lines (Bagnato et al.,1995). In breast cancer, a number of reports have demonstrated the release of immunoreactive ET-1 from MDA-MB-231, T47D, ZR-75-1 and MCF-7 breast epithelial cancer cell lines

(Suzuki et al., 1989; Kusuhara et al., 1990, Schrey et al., 1992).

From the two ovarian cancer cell lines used in our study, PEO14 cells produced ten times the amount of immunoreactive ET-1 secreted by PEO4 cells (17.5 and 1.7 fmol/10^6 cells for PEO14 and PEO4 cells respectively). The amounts of ET-like peptide released by these two cell lines were comparable to levels released from SK-OV-3 (61.2±5 fmol/10^6 cells) OVCA-433 (75.2±4 fmol/10^6 cells) and A2780 (65±3.7 fmol/10^6 cells) cell lines (Bagnato et al., 1995).

The breast epithelial cancer cell lines all produced significant amounts of ET-1-like material with T47D cells producing the greatest amounts (52±12 fmol/10^6 cells) and MDA-MB-231 and ZR-75-1 cells producing lower concentrations of ET-1-like peptide (14±3 and 17±7 fmol/10^6 cells). Values of ET-1-like peptide released from the breast epithelial cell lines were directly comparable to values mentioned in previous reports (46.4, 14 and 7.2 fmol/10^6 cells for T47D, MDA-MB-231 and ZR-75-1 cells respectively) (Suzuki et al., 1989; Kusuhara et al., 1990; Schihiri et al., 1991; Schrey et al., 1992). Such reports have also suggested that hormone-dependent breast cancer cell lines (T47D,MCF-7, ZR-75-1) secreted up to twenty times the amount of ET-1 secreted by hormone-independent cells (MDA-MB-231). In the present study, although T47D cells produced higher levels of ET-1 than the rest of the cell lines, such a large difference in ET-1 production was not observed (MDA-MB-231 and ZR-75-1 cells produced almost equal amounts). Conditioned media collected from the human ovarian (PEO12F, PEO14F and PEO27F), breast (BRF1 and BRF2) and S3T3 mouse fibroblasts were also measured by radioimmunoassay and results suggested that no significant amounts of ET-1-like material were secreted by these cells.

A number of peptides (bombesin, cortisol, oestradiol, tamoxifen, TNF, IFN-γ, IL-1ß, IL-6, TGF-ß, and prolactin) have been used as possible modulators of ET-1 production in breast cancer. Bombesin, cortisol, glucocorticoids, IL-6, have all been shown to stimulate ET-1 secretion in breast cancer cell lines (T47D, ZR-75-1, MCF-7) (Schrey et al., 1992;

Yamashita et al., 1993). The modulation of ET-1 production by these factors has not been investigated in ovarian cancer cell lines.

In this study, addition of bombesin and IL-6 to ovarian and breast epithelial cancer cells 24h prior to collection of conditioned media resulted in a stimulation of immunoreactive ET-1-like material from the cell lines.

Bombesin (100nM) stimulated ET-1-like material secretion by 40% and 400% in PEO4 and PEO14 ovarian epithelial cancer cells respectively while addition of IL-6 (200ng/ml) to the same cell lines resulted in a 38% and 310% increase in basal ET-1-like secretion (in PEO4 and PEO14 cells respectively). In breast epithelial cancer cells, bombesin stimulated an increase of 52%, 496% and 57% in the secretion of immunoreactive ET-1 detected in conditioned media collected from MDA-MB-231, T47D and ZR-75-1 cells respectively. These values are comparable to levels of stimulation reported (580%) for T47D cells (Schrey et al., 1992). Addition of IL-6 into the media of breast epithelial cancer cells also resulted in stimulation of released ET-1-like material by 26%, 80% and 37% compared to basal levels for MDA-MB-231, T47D and ZR-75-1 cells respectively. Such effects are of similar magnitude to those reported in previous studies with MCF-7 (206% increase) and ZR-75-1 (314% increase) cells (Yamashita et al., 1993). The possibility of similar results due to the cross-reactivity of bombesin and IL-6 with the ET-1 targeted antibody in the radioimmunoassay, was excluded because the radioimmunoassay failed to detect any ET-1-like material when serial dilutions of bombesin and IL-6 were assayed (figure 4.1.2.1). The potential effect of bombesin and IL-6 on the production of ET-1 from ovarian and breast cancer cell lines was investigated due to a number of observations regarding the presence and roles of these peptides in these systems.

Both bombesin and ET-1 have been reported to be present in breast cyst fluids and bombesin-related peptides have been identified at significantly higher levels in cyst fluids from women with apocrine breast cysts (Lai et al., 1990). Bombesin has also been shown to stimulate cellular signalling

in breast (Patel and Schrey 1990) and ovarian (Christen et al., 1994) cell lines, without being accompanied by mitogenic responses.

IL-6 has been detected in the systemic circulation of the local neoplastic tissue in cancer patients and has been suggested to act as the major systemic mediator of the early host response (acute phase response) to infection and injury (Sehgal et al., 1988; Kishimoto et al., 1989; Bataile et. Al., 1989). It has also been demonstrated that ET-1 plasma-levels are elevated following acute physical stress situations (Miyauchi et al., 1989) and interestingly, in the 5'-flanking region of the cloned preproendothelin-1 gene, there exist sequences of the acute phase reactant-regulatory elements which mediate the gene expression under acute physical stress in vivo (Inoue et al., 1989). Breast cancer cell lines like MCF-7 and ZR-75-1 have been shown to express high affinity IL-6 binding receptors, while ovarian cancer cell lines (OVCA) and ovarian carcinomas have been shown to contain high levels of ET-1 (Offner et al., 1995; Schroder et al., 1994) which can be regulated by other cytokines such as IL-1, TNF-α and IFN-γ (Offner et al., 1995). IL-6 levels are higher in malignant compared to benign ovarian tumours (van-der Zee et al., 1995) while the same difference has been observed with ET-1 in malignant compared to benign breast tumours (Yamashita et al., 1991). In breast cancer cells, IL-6 has also been associated with cellular morphology and it has been suggested that IL-6 treated ZR-75-1 cells convert from an epithelial to fibroblastoid shape followed by decreased cell association and increased cell motility (Tamm et al., 1989, 1991). IL-6 has been the only cytokine shown to cause the same conversion to ductal cell carcinoma cells from epithelial to fibroblast phenotype. ET-1 has been shown to induce an increase in the gene expression and synthesis of matrix proteins such as fibronectin and type IV collagen and cell proliferation through ET_A-R in messangial cells (Gomez-Garre et al., 1996).

Combining these observations with the results discussed above it could be suggested that bombesin and IL-6 could affect the production of ET-1 in an autocrine or paracrine manner. Such interaction of bombesin and IL-6

could result in increased levels of ET-1 in cancer cells with potential mitogenic effects as well as possible effects on the motility and cell-cell interaction through synergistic effects of ET-1 with IL-6.

5.1 Optimisation of the assay conditions

5.1.1 Validation of method

The assay method used for the binding experiments is described in detail in section 2.2.3 of the methods. However a series of experiments were performed initially in order to optimize the method in terms of temperature, incubation period and specificity of the assay.

(i) Influence of temperature and incubation period on specific binding

Membrane preparation samples from PEO14 cells were incubated with ^{125}I-ET-1 labelled peptide (50pM) for up to 180 min at 4°, 26° and 37°C. Triplicate samples were collected at 10, 30, 60, 90, 120 and 150 min from experiments performed at all three temperatures and binding was determined by γ-counter. Non-specific binding was determined by incubation of the same membrane preparation sample with 0.36nM of ^{125}I-ET-1 in the presence of 100-fold (0.4μM) of non-labelled ET-1. At all 3 temperatures no binding was observed in the first 30 min of incubation (figure 5.1). After that period, and until approximately 60 min of incubation time, binding increased to maximum levels in the experiments performed at 26° and 37°C with the latter temperature showing the highest binding. Incubation at 4°C resulted in less but continuously increasing binding reaching a maximum level at 120 min of incubation before plateauing. At 26° and 37°C, there was a decrease in binding between 60 and 120 min of incubation and binding reached a plateau between 120 and 150 min of incubation. From the results of these experiments, (demonstrated in figure 5.1) it was concluded that incubation of the samples for 60 min at 26°C provided the optional conditions for detection of the specific binding of ^{125}I-ET-1 to membrane preparation and these were therefore used for all the experiments.

(ii) Specificity of binding

The specificity of binding of ^{125}I-ET-1 was tested on a membrane preparation of PEO14 cells incubated with various concentrations of non-

radiolabelled related (ET-1) and unrelated peptides (bombesin and insulin). The results, shown in figure 5.2, suggest that ET-1 displaced approximately 70% of the binding while bombesin and insulin did not displace any labelled ET-1.

5.2 Scatchard analysis

Membrane preparation fractions (100µl) were incubated with a standard amount of ^{125}I-ET-1 (50pM) and increasing concentrations of unlabelled ET-1(50-9950 pM) for 90 min at 26°C. Scatchard analysis of the binding data obtained from a membrane preparation of S3T3 mouse fibroblasts was used as a positive control. Data obtained from that experiment resulted in a curve, a typical example of which is shown in figure 5.3 The graph suggested the presence of two binding sites and, in order to interpret and plot the data, the"Ligand" binding program was used. The intercept of the x-axis of the line on the left represents high affinity sites and the intercept of the line on the right represents low affinity sites. The inverse of the slopes represents the Kd.

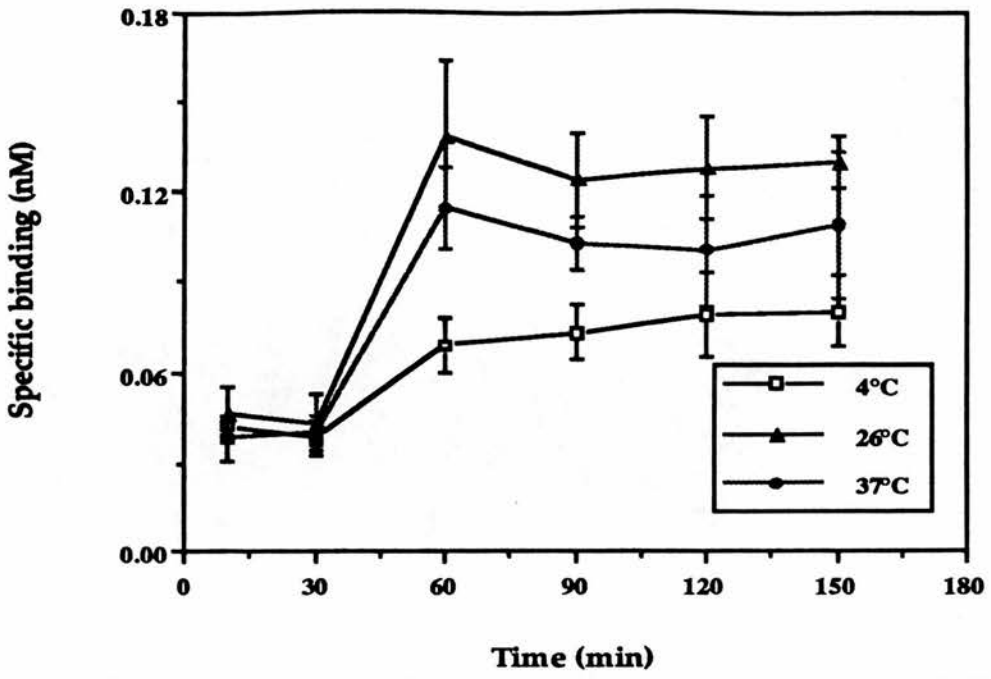


Figure 5.1: Effect of varying time and temperature of incubation period on the binding of ^{125}I-ET-1 (0.36nM) to PEO14 cell membrane preparation for up to 180min at 4°, 26° and 37°C. Non specific binding was determined by the addition of 100-fold excess unlabelled ET-1. Data represents mean values±sd of triplicate samples from a representative of 3 experiments performed on separate occasion.

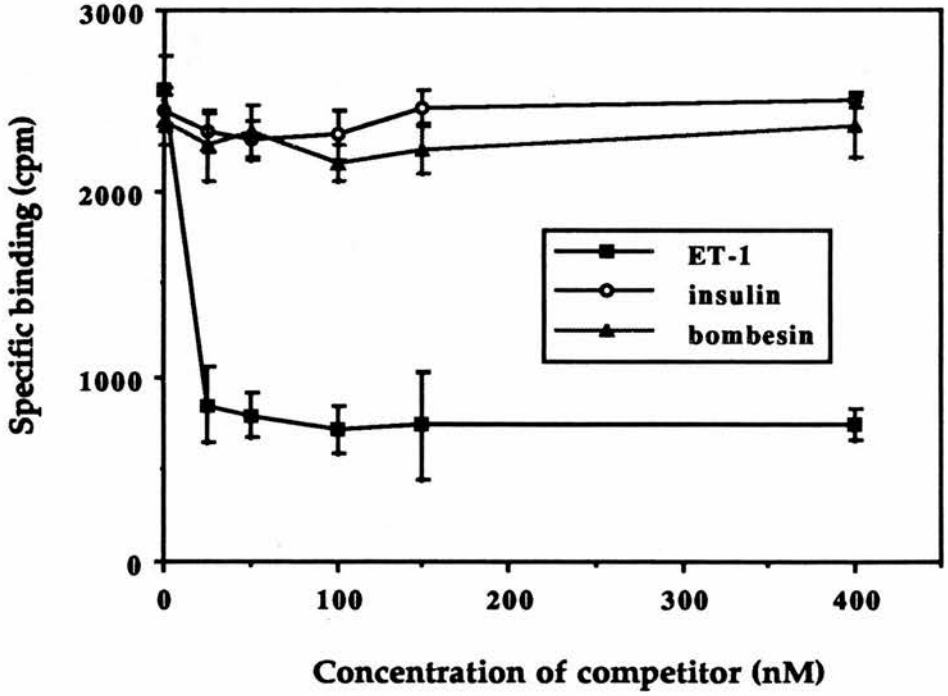


Table 5.2: Specificity of binding of ^{125}I-ET-1 to PEO14 ovarian cancer cells. A fraction of membrane preparation was incubated with 50pM of 125I-ET-1 in the presence or absence of competing peptide (25, 50, 100, 150 and 400 nM) for 60 min at 26°C. Points represent mean±se of triplicate samples from a representative of two experiments.

5.2.1 Scatchard of binding in ovarian epithelial cancer and S3T3 cells

Membrane fractions of ovarian epithelial cancer cells were prepared from PEO4, PEO14 and S3T3 (positive control) cell lines growing in phenol red-free, serum-free DMEM culture media containing HITS (hydrocortisone, insulin, transferrin and sodium selenite).

(i) S3T3 fibroblasts

The S3T3 fibroblasts were used as a positive control in this set of experiments. Scatchard analysis (figure 5.3) indicated the presence of two distinct binding sites. A higher affinity binding site with a dissociation constant of 0.9 nM (Bmax: 0.49 pmol/mg of protein) and a lower affinity binding site with a dissociation constant of 7.10 nM (Bmax: 1.68 pmol/mg of protein).

(ii) PEO4 cells

For PEO4 cells (figure 5.4), the Scatchard analysis produced a plot which suggested the presence of two distinct classes of receptor binding sites. The higher affinity receptor site had a dissociation constant value of 0.065 nM (Bmax: 0.047 pmol/mg of protein), whereas the lower affinity receptor site had a dissociation constant value of 0.49nM (Bmax: 0.23 pmol/mg of protein).

(iii) PEO14 cells

For the PEO14 cell line, the Scatchard analysis indicated the presence of a single class of lower affinity receptor binding site (figure 5.5) with a dissociation constant value of 0.56 nM (Bmax: 0.31 pmol/mg of protein).

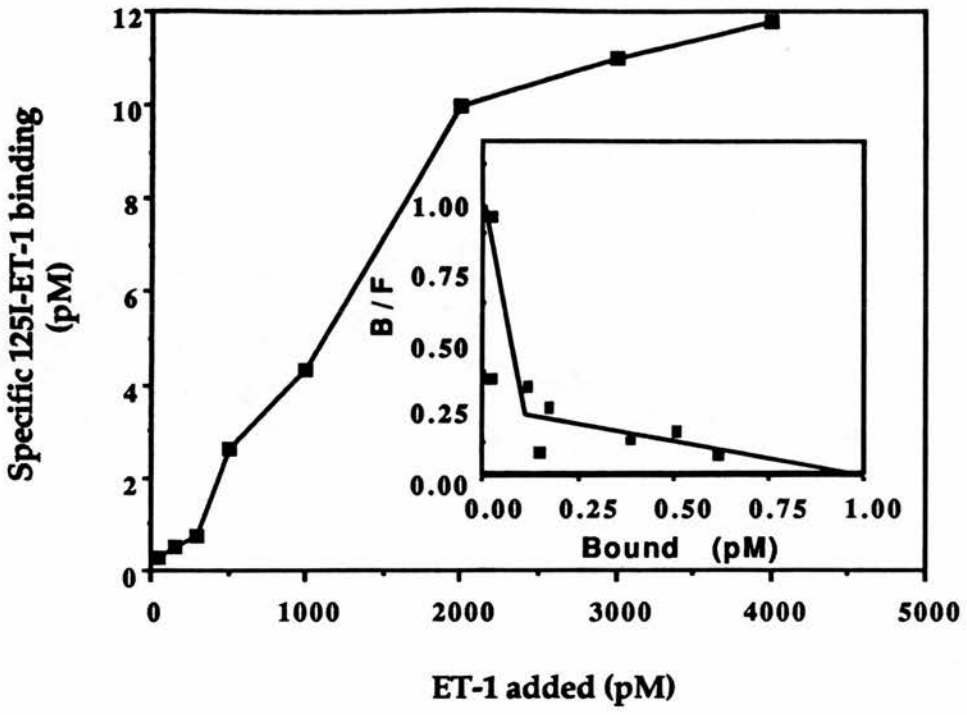


Figure 5.3: Saturation curve of the binding of ^{125}I-ET-1 to S3T3 mouse fibroblasts. Increasing concentrations of ET-1 were added to membranes prepared from cells in duplicate in the presence (non-specific) or absence (total binding) unlabelled (1μM) ET-1. Cells were incubated at 26°C for 60min. Scatchard analysis results were plotted with values obtained by "LIGAND" computer program and represent a typical result of 3 experiments performed on separate occasions.

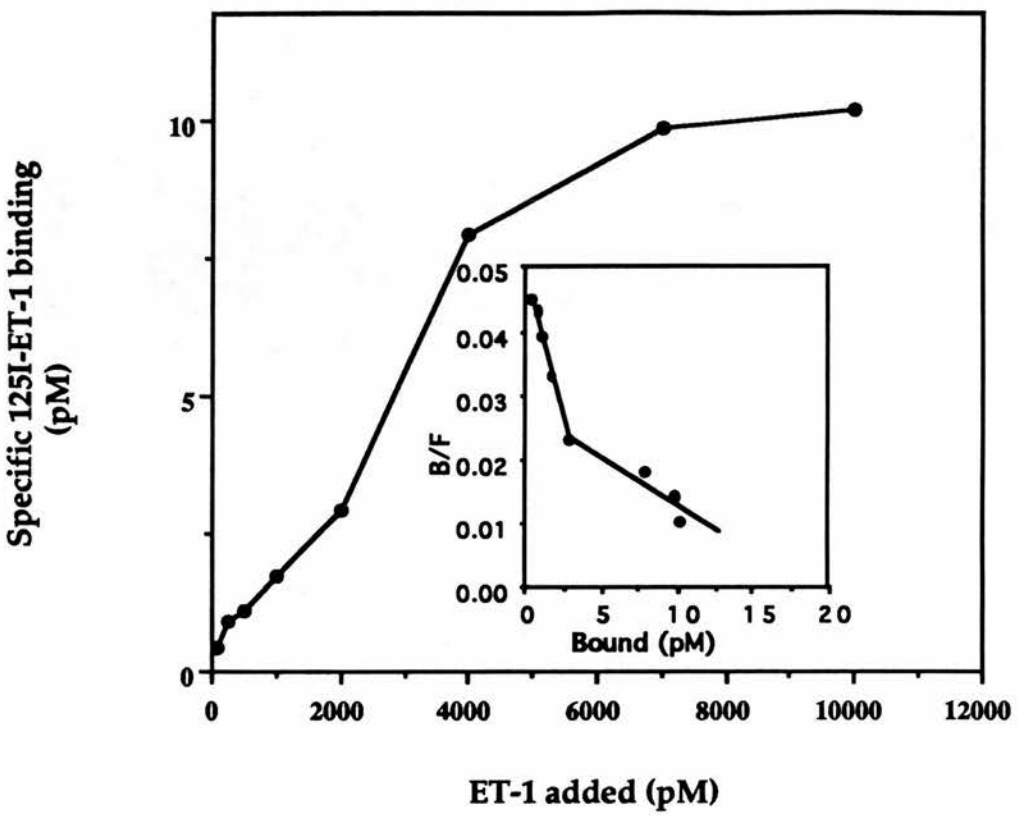


Figure 5.4: Saturation curve of the binding of ^{125}I-ET-1 to PEO4 ovarian cancer cells. Increasing concentrations of ET-1 were added to membranes prepared from cells in duplicate in the presence (non-specific) or absence (total binding) unlabelled (1μM) ET-1. Cells were incubated at 26°C for 60min. Scatchard analysis results were plotted with values obtained by "LIGAND" computer program and represent a typical result of 3 experiments performed on separate occasions.

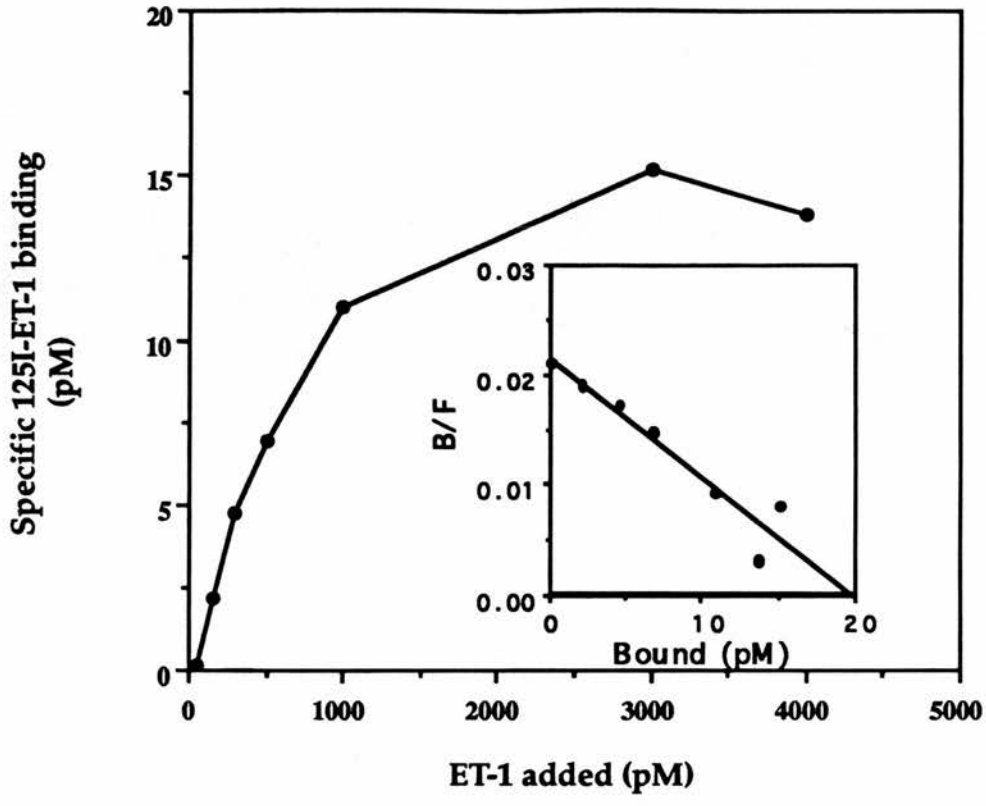


Figure 5.5: Saturation curve of the binding of ^{125}I-ET-1 to PEO14 ovarian cancer cells. Increasing concentrations of ET-1 were added to membranes prepared from cells in duplicate in the presence (non-specific) or absence (total binding) unlabelled (1μM) ET-1. Cells were incubated at 26°C for 60min. Scatchard analysis results were plotted with values obtained by "LIGAND" computer program and represent a typical result of 3 experiments performed on separate occasions.

5.2.2 Scatchard analysis of binding in human breast epithelial cancer cells

Membrane fractions of two breast epithelial cancer cell lines, T47D and MDA-MB-231, were prepared and used in binding experiments. Results shown in figure 5.6 demonstrate the background level of binding of ^{125}I-ET-1 with membrane preparations from the two breast cancer cell lines, suggesting the absence of ET receptors in MDA-MB-231 and T47D cells.

5.3 Binding inhibition experiments

For the binding inhibition studies, cell membrane samples (100µl) were incubated with ^{125}I-ET-1 (50pM) and increasing concentrations (10pM to 1µM) of ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Samples were incubated at 26°C for 90 min and counted in a γ-counter. Non specific binding was determined in the presence of 1µM ET-1.

5.3.1 Ovarian cancer cells

Membrane preparations of PEO4 and PEO14 cell lines were used in binding inhibition experiments in order to determine the specific receptor subtype expressed in these cells.

(i) PEO4 cells

A typical experiment using membrane preparations from PEO4 cells is shown in figure 5.7. The half maximal inhibitory concentrations for ET-1, ET-3, BQ123 and BQ788 were 0.04, 3, 0.2 and >1000 nM respectively. These results suggested the predominant expression of ET_A-R in these cells. However, a small inhibition of ^{125}I-ET-1 binding in the presence of BQ788 (ET_B-R) antagonist suggested the expression of a limited amount of ET_B receptors in PEO4 cells.

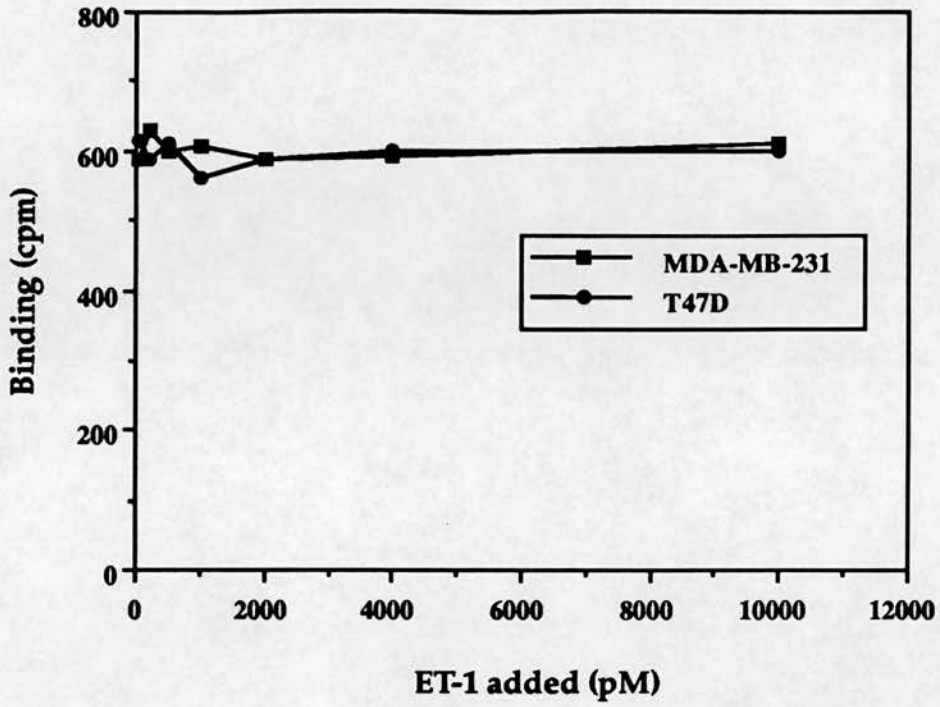


Table 5.6: Binding (cpm) of ET-1 to membrane preparations of MDA-MB-231 and T47D breast cancer cells. Membrane samples were incubated with increasing concentrations of ^{125}IET-1 (50, 100, 250, 500, 1000, 2000, 4000 and 10000pM) at 26°C for 60 min. Figure shows results of a representative of two experiments performed on separate occasions.

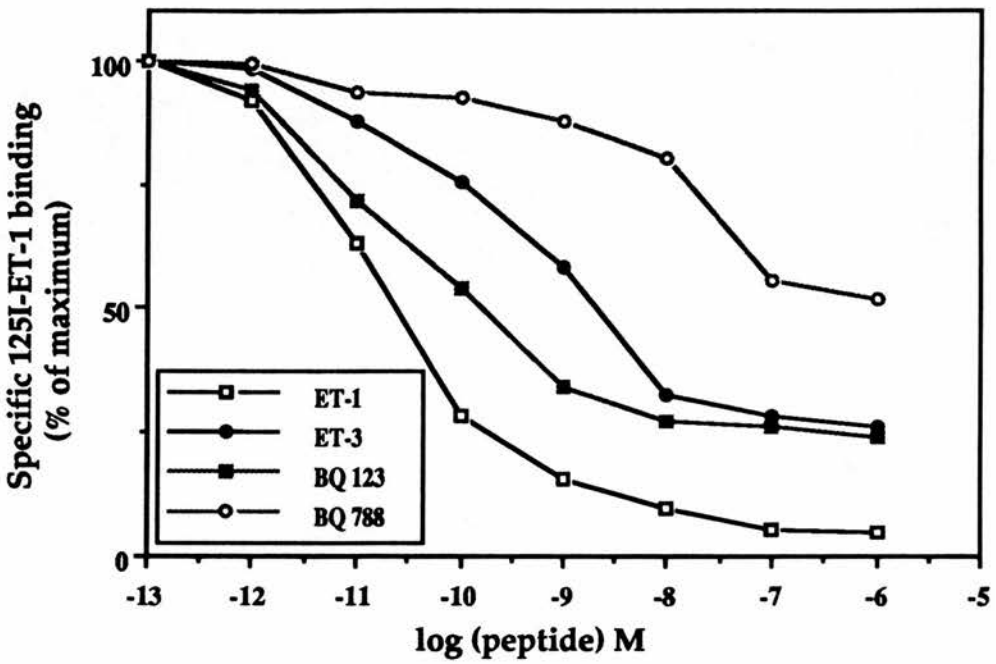


Figure 5.7: Inhibition of specific ^{125}I-ET-1 binding in PEO4 ovarian cancer cell membrane preparations by ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Cell membranes were incubated at 37°C for 60min with 50pM ^{125}I-ET-1 in the presence or absence of the above peptides at the concentration indicated. Graph presents results of a representative of 3 experiments performed on separate occasions. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

(ii) PEO14 cells

For PEO14 cells, (figure 5.8) half maximal inhibitory concentrations for ET-1, ET-3, BQ123 and BQ788 were 0.17, 10, 0.85 and >1000 nM respectively. Although as in PEO4 cells, the maximal inhibitory concentration of BQ788 antagonist was >1000 nM, there was no change in the pattern of the effect even at very high concentrations. Therefore, and in contrast to what was observed in PEO4 cells, results suggest the expression of ET_A-R only in PEO14 cells.

5.3.2 Ovarian fibroblasts

Membrane fractions from PEO12F fibroblasts were prepared for binding experiments. The limited growth of these cells precluded Scatchard analysis however, sufficient numbers were available for binding inhibition studies. As shown in figure 5.9, half maximal inhibitory concentrations for ET-1, ET-3, BQ123 and BQ788 were 3, 20, 140 and 500nM respectively. These results suggest the expression of both ET_A-R and ET_B-R in PEO12F ovarian fibroblasts.

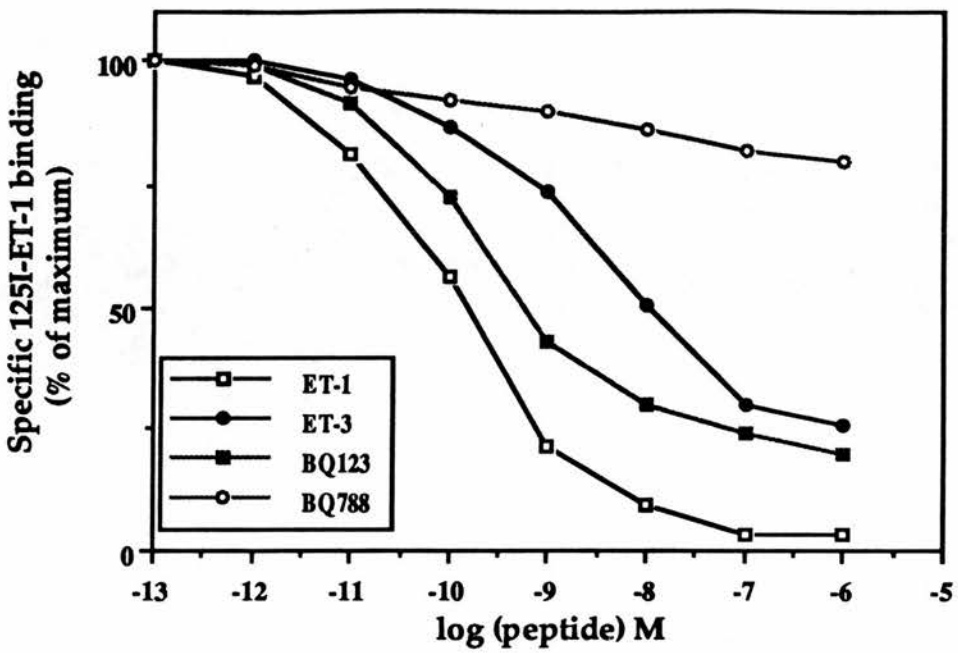


Figure 5.8: Inhibition of specific ^{125}I-ET-1 binding in PEO14 ovarian cancer cell membrane preparations by ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Cell membranes were incubated at 37°C for 60min with 50pM ^{125}I-ET-1 in the presence or absence of the above peptides at the concentration indicated. Graph presents results of a representative of 3 experiments performed on separate occasions. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

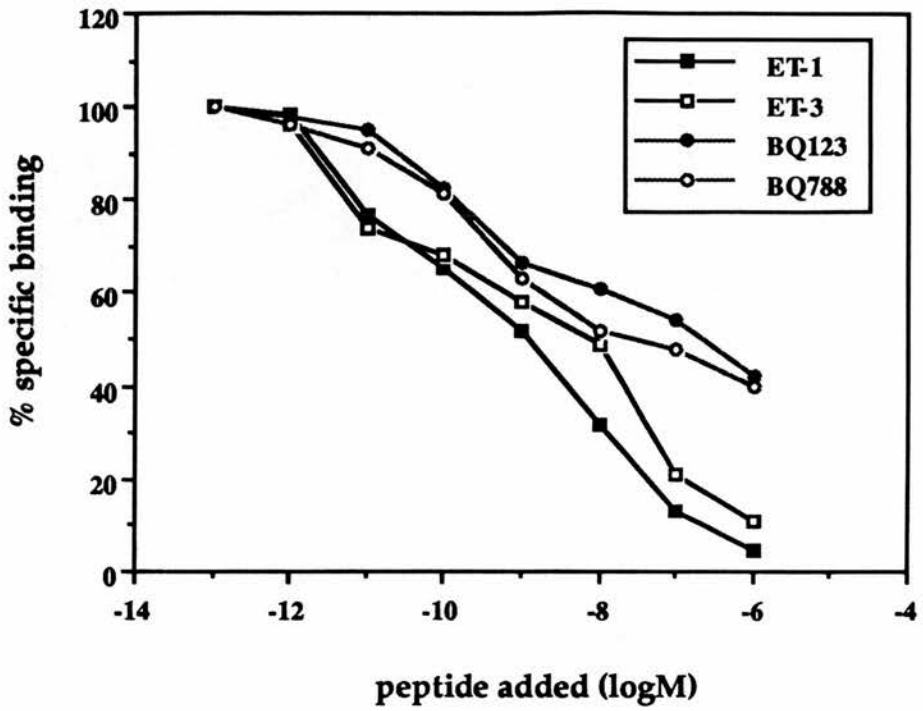


Figure 5.9: Inhibition of specific ^{125}I-ET-1 binding in PEO12F ovarian fibroblast membrane preparations by ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Cell membranes were incubated at 37°C for 60min with 50pM ^{125}I-ET-1 in the presence or absence of the above peptides at the concentration indicated. Graph presents results of a representative of 3 experiments performed on separate occasions. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

5.3.3 Breast fibroblasts

As with PEO12F cells, BRF1 cells were cultured in order to prepare membranes for use in binding inhibition experiments. As shown in figure 5.10, ET-1 and ET-3 and both antagonists inhibited the ^{125}I-ET-1 specific binding. Half maximal inhibitory concentrations were 2.5nM for ET-1, 7nM for ET-3, 100nM for BQ123 and 900nM BQ788. Results suggest the expression of both ET_A-R and ET_B-R in BRF1 breast fibroblasts.

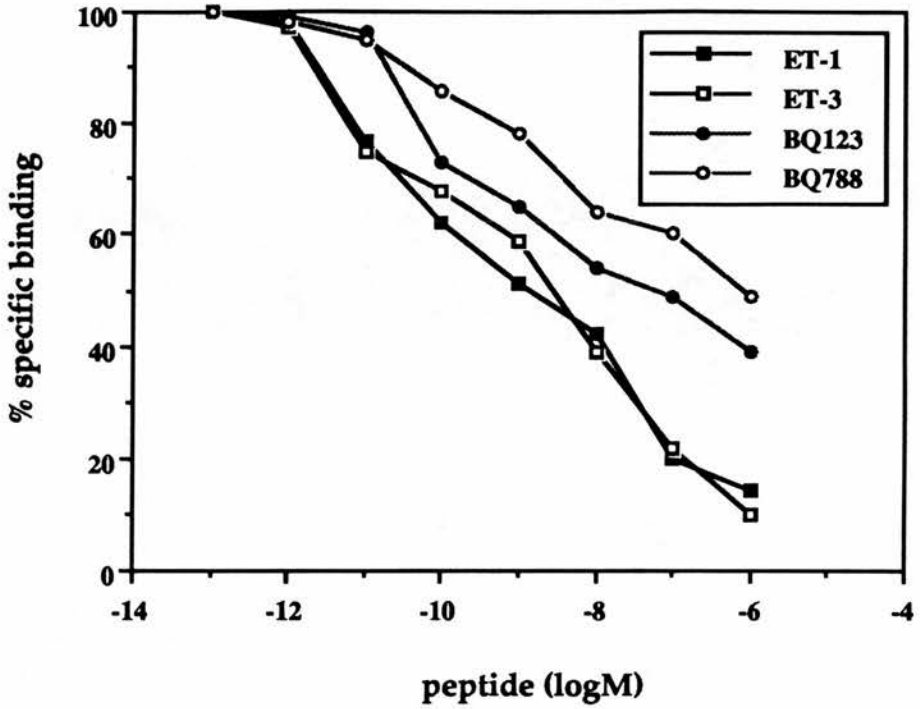


Figure 5.10: Inhibition of specific ^{125}I-ET-1 binding in BRF1 breast fibroblast membrane preparations by ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist). Cell membranes were incubated at 37°C for 60min with 50pM ^{125}I-ET-1 in the presence or absence of the above peptides at the concentration indicated. Graph presents results of a representative of 3 experiments performed on separate occasions. Binding of ^{125}I-ET-1 is expressed as the percentage of the specific binding in the absence of unlabelled peptide.

5.4 Discussion

Results presented in this chapter suggest that while breast epithelial cancer cell lines MDA-MB-231, T47D and ZR-75-1 do not bind ^{125}I-ET-1, ovarian epithelial cancer cell lines PEO4 and PEO14 as well as ovarian (PEO12F) and breast (BRF1) fibroblasts bind endothelin with high affinity consistent with the presence of ET-receptors.

In this study, ^{125}I-ET-1 exhibited specific binding with membrane preparations of both PEO4 and PEO14 ovarian epithelial cancer cells. Results from PEO14 cells, suggested the presence of a single type of receptor binding site (Kd: 0.56nM, Bmax: 0.31pmol/mg of protein) while studies on PEO4 cells suggested the presence of two distinct binding sites, a lower affinity binding site (Kd: 0.49nM, Bmax: 0.23pmol/mg of protein) similar to the one seen in PEO14 cells, and a higher affinity binding site (Kd: 0.065nM, Bmax: 0.047pmol/mg protein).

As has already been suggested (Kenakin et al., 1992; Huggins, 1993) the identification of the specific type of receptors present in a system could not be entirely based on binding data due to the similarities of Kd's for the two major types of ET receptors. There are also suggestions about the presence of two subtypes with Kd's of the pM and nM range for each of the ET_A and ET_B receptors (Ambar and Sokolovsky., 1993; Simonson and Rooney., 1993). Reports on a third type of endothelin receptor ET_C with a proposed rank order of affinity of ET-3>ET-1 added further complication (Karne et al., 1993). Therefore, and unless in situations where tissues represent pure sources of receptors subtype (e.g. rat aorta, for ET_A-R, Huggins at al., 1993), binding inhibition curves could provide further information when carefully analysed.

The nature of the receptors expressed on the surface of both cell lines was investigated in competition experiments where ET-1, ET-3, BQ123 (ET_A-R antagonist) and BQ788 (ET_B-R antagonist) were competing with ^{125}I-ET-1 for binding to membrane preparations from both cell lines. For PEO4 cells, specific ^{125}I-ET-1 binding to membrane preparations was inhibited by the presence of increased concentrations of BQ123 antagonist and to a

lesser extent by addition of ET-3. The addition of increased concentrations of BQ788 had a small effect at very high concentrations. For PEO14 cells, ^{125}I-ET-1 binding was inhibited by increasing BQ123 concentrations and less affected by ET-3 addition but was not affected at all by the addition of BQ788 antagonist.

A single study by Bagnato et al. (1995) on 3 ovarian epithelial cancer cell lines (SKOV-3, A2780 and OVCA-433) has suggested the expression a single type of ET receptor with Kd values of 0.15, 0.03 and 0.02 nM respectively and specific binding was blocked with the addition of BQ123 suggesting that receptors were of the ET_A-R type.

Combining the results from this study it could be suggested that PEO14 cells express a single type of receptors (Kd: 0.56nM) which can be blocked with BQ123 antagonist and therefore is of the ET_A-R type. For PEO4 cells however, two receptor binding sites have been observed. One with a Kd of 0.49nM very similar to that seen with PEO14 cells and a second higher affinity binding site (Kd: 0.065 nM). This higher affinity binding site could represent higher affinity ET_A also seen in S3T3 cells (Ambar and Sokolovsky 1992), or high affinity ET_B receptors. Analysing the results of the binding inhibition experiments for PEO4 it can be suggested that the major type of receptors expressed is that of ET_A-R type because of the inhibition of binding seen with the addition of BQ123. However, the small inhibition of ^{125}I-ET-1 binding in the presence of BQ788 might suggest the presence of a limited amount of ET_B receptors.

Binding inhibition experiments using membrane preparations of PEO12F ovarian fibroblast cell lines, showed that ^{125}I-ET-1 binding could be inhibited with the addition of increasing concentrations of ET-3, BQ123 and BQ788 suggesting the presence of both ET_A-R and ET_B-R in these cells.

In breast cancer, studies have suggested the absence of ET receptors in the epithelial cancer cell lines and the specific binding of ^{125}I-ET-1 to ET receptors in breast fibroblasts (Kd of 0.09nM), (Baley et al., 1990). However, there exist no further information regarding the type of ET receptors expressed in these cells.

In our study, ^{125}I-ET-1 showed no specific binding to membrane preparations from MDA-MB-231, T47D and ZR-75-1 cells which is consistent with the absence of ET receptors in breast epithelial cancer cells. However, binding inhibition experiments have suggested the presence of such receptors in breast fibroblasts. In BRF1 cells, increasing concentrations of ET-3, BQ123 and BQ788 all inhibited the binding of ^{125}I-ET-1 suggesting the expression of both types of ET receptors in these cells. In summary, results shown in this chapter have suggested the expression of specific ET receptors in ovarian epithelial and ovarian and breast fibroblast cell lines. Combining these results with the production of immunoreactive ET-1-like material (as measured by radioimmunoassay) from ovarian and breast cancer cell lines it could be suggested that in the ovarian system endothelins secreted by the cancer cells can bind to receptors expressed in both cancer and fibroblast cells. Therefore ETs can exert their effects in an autocrine and/or paracrine fashion. In the breast system however, ET-1-like material secreted by breast cancer cells could bind specific ET receptors expressed in breast fibroblast cells and therefore exert their effects in a paracrine manner.

Chapter 6: Effect of endothelin on the growth of ovarian and breast epithelial cancer and fibroblast cell lines

To investigate the effects of ETs on the growth of human ovarian and breast epithelial cancer and fibroblast cell lines, ETs were added exogenously to the culture media of cell lines at a range of concentrations (10^{-6} to 10^{-13} M). Cells were grown in media supplemented with 1% FCS and were exposed to ETs for 5 days. Cell numbers were compared to those of cells grown in identical conditions and for the same incubation period without being exposed to ETs.

6.1. Effect of exogenous ET addition on the growth of human ovarian epithelial cancer cell lines

The effects of endothelins (ET-1, ET-2 and ET-3) on the growth of ovarian cancer cells were investigated by adding ETs to cultures of two human ovarian cancer cell lines, PEO4 and PEO14. Results shown in figures and tables represent values from one of three experiments which produced similar results. All concentrations described below as having growth effects produced statistically significant differences from control culture without the addition of endothelins.

(i) Effects of ET-1

As shown in figure 6.1, concentrations of ET-1 between 10^{-11} and 10^{-10}M added to PEO4 cells resulted in an increasing growth stimulatory effect reaching greater effects at 10^{-10}M (153% control growth) while the degree of stimulation progressively decreased at concentrations of 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M of ET-1.

In PEO14 cells, a similar pattern of effects was detected (figure 6.1). Increasing effects were seen between concentrations of 10^{-13} and 10^{-10}M, maximum stimulation of growth was observed at 10^{-9}M (154% control growth) and decreasing effects at 10^{-8}, 10^{-7} and 10^{-6}M of added ET-1.

Table 6.1 summarises the effects of ET-1 addition between 10^{-13} and 10^{-6}M expressed as % control growth of both ovarian cancer cell lines.

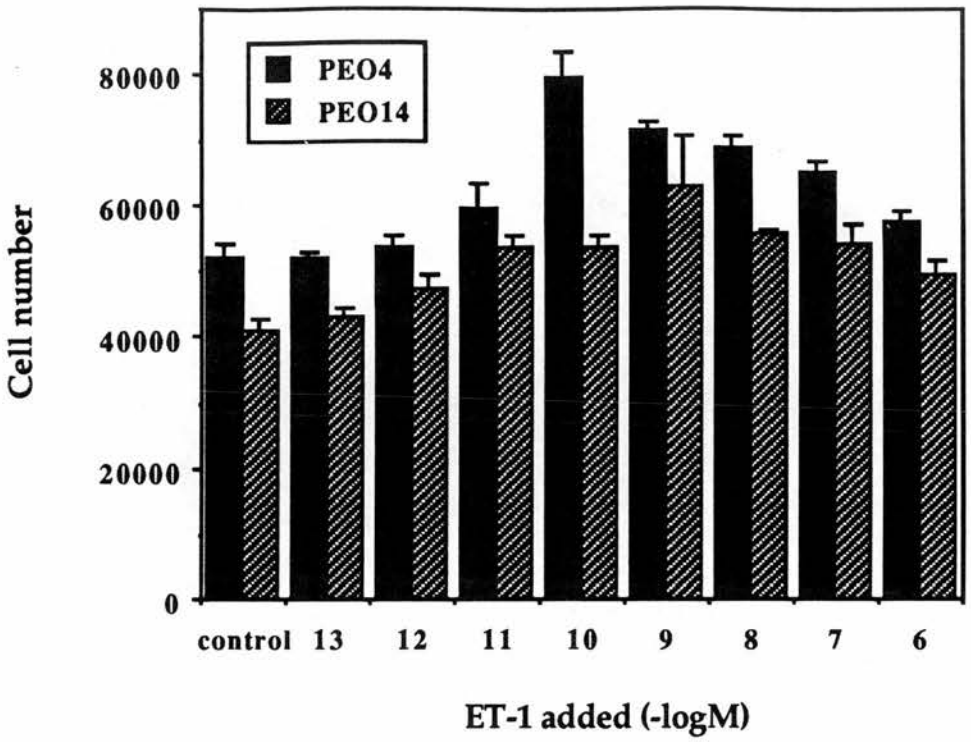


Figure 6.1: Effects of the exogenous addition of ET-1 on PEO4 and PEO14 ovarian cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-1 on the growth of ovarian cancer cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO4	% control growth	99	103	* 114	* 153	* 137	* 132	* 125	* 111
	±se	1	2.1	2.1	2.3	5.1	9.3	9.2	2.1
PEO14	% control growth	* 105	* 115	* 131	* 154	* 136	* 136	* 133	* 120
	±se	1.1	2.6	6.1	8.5	4.2	2.2	4.1	1.7

Table 6.1. The effects of exogenous endothelin-1, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO4 and PEO14 ovarian carcinoma cells expressed as % control cell number ($5.2x10^4$±1432 and $4.1x10^4$±1011 for PEO4 and PEO14 respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

(ii) Effect of ET-2

The effect of the addition of ET-2 on the growth of PEO4 and PEO14 cells is shown in figure 6.2.

In PEO4 cells, addition of ET-2 at concentrations between 10^{-9} and 10^{-7}M resulted in an increase in control growth. Stimulation of growth reached maximum effects between 10^{-8} and 10^{-7}M (120% control growth). A lower effect was observed at 10^{-6}M of added ET-2.

In PEO14 cells, ET-2 stimulated growth, and effects were increased with 10^{-12}M, 10^{-11}M and 10^{-10}M of added ET-1, maximum effects occurring with 10^{-9}M (133%); thereafter effects with 10^{-8}M, 10^{-7}M and 10^{-6}M concentrations produced decreasing responses.

Details on the % control growth of PEO4 and PEO14 cells with the addition of ET-2 are presented in table 6.2.

(iii) Effect of ET-3

As shown in figure 6.3, results of the exogenous addition of ET-3 at concentrations of 10^{-13} to 10^{-6}M did not have significant effects on the growth of PEO4 and PEO14 cells in culture. Table 6.3 summarises all the results in the form of % control cell number.

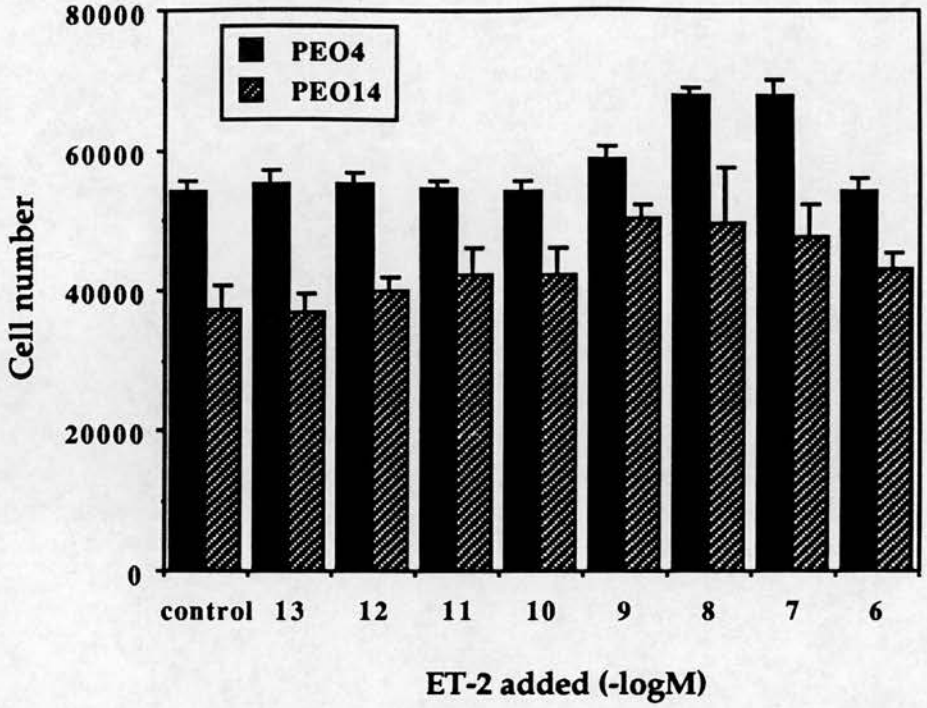


Figure 6.2: Effects of the exogenous addition of ET-2 on PEO4 and PEO14 ovarian cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-2 on the growth of ovarian cancer cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO4	% control growth	101	102	102	98	* 105	* 119	* 120	102
	±se	1.9	2.2	4.3	3.6	1.9	3.1	5	4.8
PEO14	% control growth	99	* 107	* 113	* 113	* 133	* 131	* 127	* 115
	±se	2	3.4	4.7	3.1	2.9	5.5	3.2	4.3

Table 6.2. The effects of exogenous endothelin-2, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO4 and PEO14 ovarian carcinoma cells expressed as % control cell number ($5.4x10^4$±3024 and $3.79x10^4$±4014 for PEO4 and PEO14 respectively. Each value represents mean ± sd of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

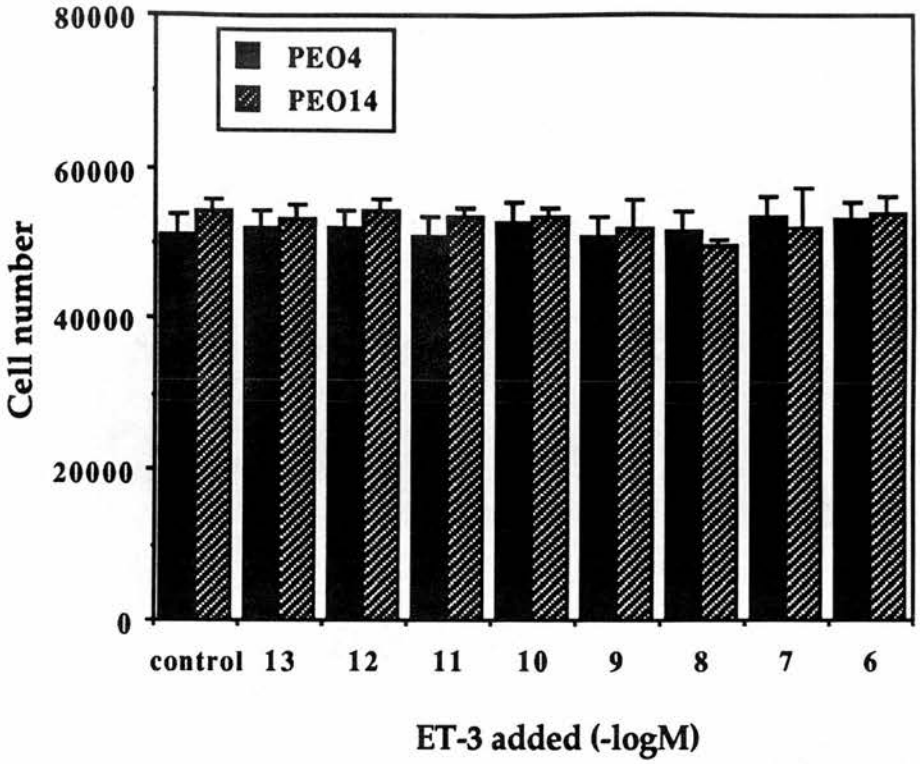


Figure 6.3: Effects of the exogenous addition of ET-3 on PEO4 and PEO14 ovarian cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-3 on the growth of ovarian cancer cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO4	% control growth	101	101	98	103	99	100	104	103
	±se	5	3	5	6	5	8	4.4	3.7
PEO14	% control growth	96	99	98	98	97	99	100	100
	±sde	8.3	2.1	3.5	4.5	3.5	2.7	4.1	1.2

Table 6.3. The effects of exogenous endothelin-3, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO4 and PEO14 ovarian carcinoma cells expressed as % control cell number (5.1x104±2017 and 5.4x104±1001 for PEO4 and PEO14 respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

6.2 Effect of exogenous ET addition on the growth of human ovarian fibroblasts

The growth stimulatory effects of ETs on human ovarian fibroblasts were investigated with the exogenous addition of ETs to PEO12F, PEO14F and PEO27F cell lines. Results shown below comprise values obtained from a representative of 3 experiments performed on separate occasions and concentrations of ETs discussed as having effects on growth of the cell lines represent statistically significant differences compared to controls.

(i) Effect of ET-1

Figure 6.4 diagrammatically presents the effects of ET-1 addition on the growth of ovarian fibroblast cell lines.

In PEO12F ovarian fibroblasts, stimulatory effects were detected at concentrations of 10^{-12}M to 10^{-9}M of ET-1 (figure 6.4). Increasing stimulatory effects were seen at concentrations of 10^{-12}M and 10^{-11}M, maximum effects were observed at 10^{-10}M (161%) and progressively lower effects at 10^{-9}M and 10^{-6}M. Exogenous additions of ET-1 at concentrations of 10^{-13}, 10^{-8} and 10^{-7}M had no significant effect on the growth of the cells.

In PEO14F cells, growth stimulatory effects of the exogenous ET-1 addition were detected at a wider range of concentrations compared to PEO12F (figure 6.4). Addition of ET-1 at concentrations between 10^{-13} and 10^{-11} M resulted in increasing growth stimulatory effects reaching maximum levels at 10^{-10}M (164% control cell number) while addition of 10^{-9}, 10^{-8} and 10^{-7}M resulted in decreasing stimulatory effects.

In PEO27F fibroblasts (figure 6.4) the addition of ET-1 between 10^{-13} and 10^{-6}M exerted growth stimulatory effects. The pattern of effects was similar to PEO12F and PEO14F fibroblasts with increasing stimulation of growth seen between concentrations of 10^{-13} and 10^{-10}M, maximum effects at 10^{-10}M (166%) and decreasing growth stimulatory effects between 10^{-9}M and 10^{-6}M of added ET-1.

Table 6.4 summarises the effects of ET-1 in all 3 fibroblast cell lines.

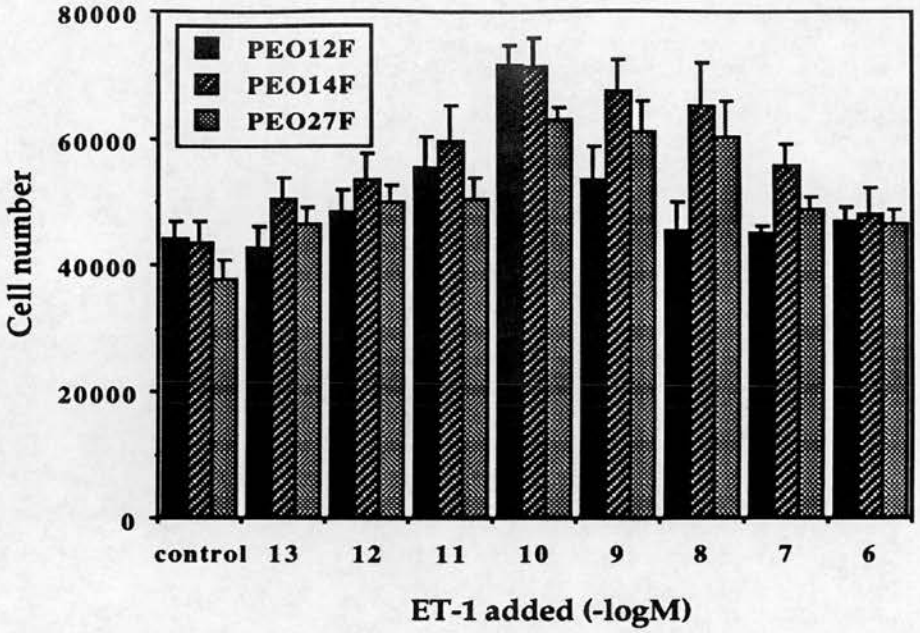


Figure 6.4: Effects of the exogenous addition of ET-1 on PEO12F, PEO14F and PEO27F ovarian fibroblasts growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-1 on the growth of ovarian fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO12F	% control growth	96	* 109	* 124	* 161	* 120	102	101	* 106
	±se	5.3	7.9	11.2	6.5	12.4	10.5	4.8	2.9
PEO14F	%control growth	* 116	* 123	* 137	* 164	* 155	* 150	* 128	111
	±se	7.6	9	12.6	9.9	11.5	15.5	7.6	9.3
PEO27F	% control growth	* 123	* 132	* 133	* 166	* 161	* 159	* 129	* 123
	±se	6.5	6.9	8.4	4.3	12.3	14.6	4.5	5.3

Table 6.4. The effects of exogenous endothelin-1, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO12F, PEO14F and PEO27F ovarian fibroblast cell lines expressed as % control cell number ($4.3x10^4$±2432, $4.2x10^4$±3387, $3.8x10^4$±3102 for PEO12F, PEO14F and PEO27F respectively). Each value represents mean ± sd of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

(ii) Effect of ET-2

The effects of ET-2 addition on the growth of ovarian fibroblast cell lines are shown in figure 6.5.

In PEO12F cells, exogenous ET-2 addition stimulated growth at concentrations between 10^{-12} and 10^{-6}M. Increasing effect were observed between 10^{-12} and 10^{-8}M, maximum effects were reached with the addition of 10^{-8}M (199% control growth), while higher concentrations of added ET-2 (10^{-7} and 10^{-6}M) resulted in decreasing stimulatory effects.

In the same figure it can be observed that ET-2 addition to PEO14F fibroblast cells had similar effects to those observed in PEO12F fibroblasts; stimulatory effects were observed between concentrations of 10^{-12} to 10^{-6} M. Increasing effects were observed between concentrations of 10^{-12} and 10^{-10}M, maximum effects with 10^{-9}M (160% control growth) and decreasing effects with 10^{-8}, 10^{-7} and 10^{-6}M.

In PEO27F cells, stimulatory effects were seen between concentrations of 10^{-13} and 10^{-7}M (figure 6.5). Increasing levels of growth stimulation were detected between 10^{-13} and 10^{-10}M of ET-2, maximum effects with the addition of 10^{-9}M (164% control growth) and decreasing stimulation of growth with 10^{-8} and 10^{-7}M ET-2 addition.

Table 6.5 summarises the effects of ET-2 on all 3 cell lines expressed as %control cell number.

(iii) Effect of ET-3

The effects of ET-3 on the growth of ovarian fibroblast cell lines were also investigated and are shown in figure 6.6.

In PEO12F cells, stimulation of growth was observed with the addition of ET-3 at concentrations between 10^{-12} and 10^{-6}M. More precisely, concentrations between 10^{-12} and 10^{-10}M resulted in increasing stimulation of growth (maximum effect seen at 10^{-10}M was 175% control cell number). Decreasing effects were observed between concentrations of 10^{-9} and 10^{-6}M.

In the same figure, stimulatory effects were seen for PEO14F cells at concentrations between 10^{-12} and 10^{-7}M. Within this range of

concentrations, increasing stimulatory effects were observed at 10^{-12}, 10^{-11} and 10^{-10}M, maximum effects at 10^{-9}M (153% control growth) and decreasing effects at 10^{-8} and 10^{-7}M.

In PEO27F fibroblasts, growth stimulatory effects were observed between concentrations of 10^{-12} and 10^{-7}M of exogenous ET-3 (figure 6.5). The pattern of the effects was similar to that observed in PEO12F and PEO27F fibroblasts with increasing stimulatory effects between 10^{-12} and 10^{-10}M, maximum effects at 10^{-9} and 10^{-8}M (158% and 159% control growth, respectively) and decreasing stimulatory effects at 10^{-7} and 10^{-6}M.

Table 6.6 summarises all the results of the effects of ET-3 in ovarian fibroblasts cell lines, expressed as % control cell numbers.

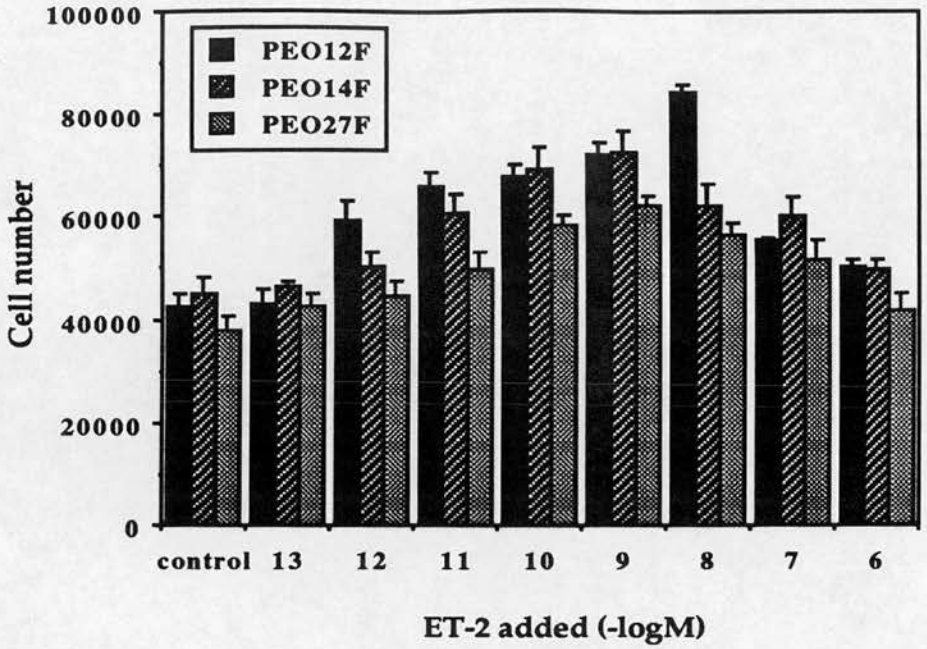


Figure 6.5: Effects of the exogenous addition of ET-2 on PEO12F, PEO14F and PEO27F ovarian fibroblast cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-2 on the growth of ovarian fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO12F	% control growth	102	* 139	* 155	* 160	* 169	* 199	* 130	* 118
	±se	5.8	9.2	6.3	4.8	6.6	2.9	2.1	3.5
PEO14F	% control growth	103	* 111	* 134	* 153	* 160	* 137	* 133	* 109
	±se	2.2	6	9	9.7	9.3	9.5	8.5	3.9
PEO27F	% control growth	* 112	* 118	* 131	* 154	* 164	* 148	* 136	110
	±se	6.7	6.7	8.4	5.2	4.6	7.3	9.5	9

Table 6.5. The effects of exogenous endothelin-2, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO12F, PEO14F and PEO27F ovarian fibroblast cell lines expressed as % control cell number ($4.1x10^4$±2034, $4.2x10^4$±3057, $3.9x10^4$±3092 for PEO12F, PEO14F and PEO27F respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

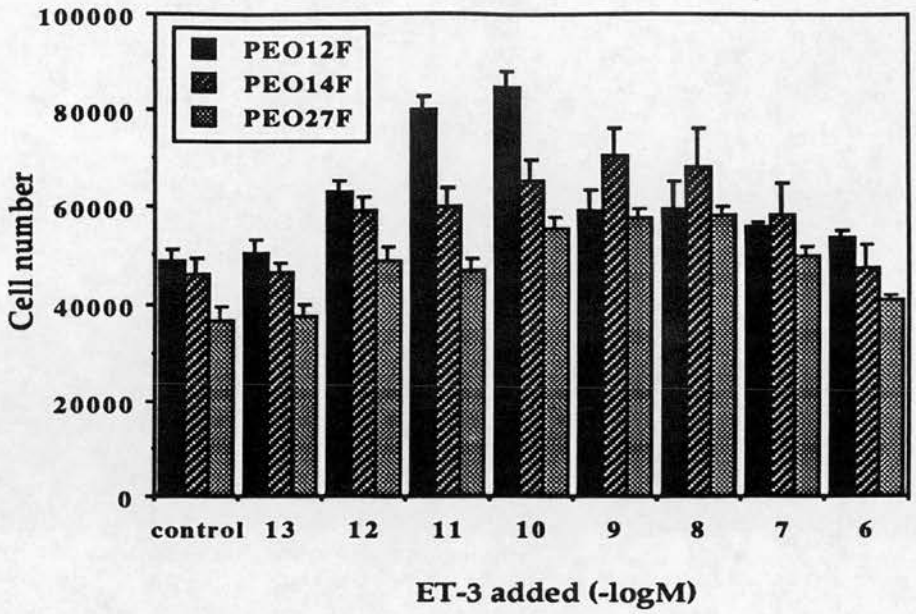


Figure 6.6: Effects of the exogenous addition of ET-3 on PEO12F, PEO14F and PEO27F ovarian fibroblast cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-3 on the growth of ovarian fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
PEO12F	% control growth	103	* 130	* 165	* 175	* 122	* 123	* 115	* 110
	±se	5.8	4.9	6.1	6.5	8.8	11.6	1.7	3
PEO14F	% control growth	101	* 129	* 131	* 142	* 153	* 148	* 127	103
	±se	4.6	6.4	8.6	9.7	12.5	17.9	14.6	10
PEO27F	% control growth	102	* 133	* 129	* 152	* 158	* 159	* 136	* 112
	±sde	6.6	8.9	5.4	5.8	5.4	5.3	5.5	2.6

Table 6.6. The effects of exogenous endothelin-3, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of PEO12F, PEO14F and PEO27F ovarian fibroblast cell lines expressed as % control cell number ($4.9x10^4$±1994, $4.8x10^4$±2397, $3.8x10^4$±1095 for PEO12F, PEO14F and PEO27F respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

6.3 Effect of exogenous ET addition on growth of human breast epithelial cancer cell lines.

The effects of the exogenous addition of ETs were investigated in the culture of three human breast epithelial cancer cell lines MDA-MB-231, T47D and ZR-75-1. Results discussed below are representative values from one of 3 experiments performed on separate occasions and with similar results.

(i) Effects of ET-1

The effects of ET-1 addition on the growth of breast cancer cell lines are shown in figure 6.7. For all three breast epithelial cancer cell lines in culture, addition of ET-1 at a range of concentrations (10^{-13} to 10^{-6} M) had no effect on the growth of the cells, since no significant difference in cell numbers was detected between treated and control cells.

(ii) Effects of ET-2

Figure 6.8 diagrammatically presents the effect of ET-2 addition on the growth of MDA-MB-231, T47D and ZR-75-1 cells. As with the addition of ET-1, the exogenous addition of ET-2 and ET-3 did not change the number of cells at the end of 5 days incubation period for the treated compared to control cells.

(iii) Effects of ET-3

Addition of ET-3 to the culture of the 3 breast cancer cell lines at a range of concentrations had no effect on the growth of the cells as shown in figure 6.9.

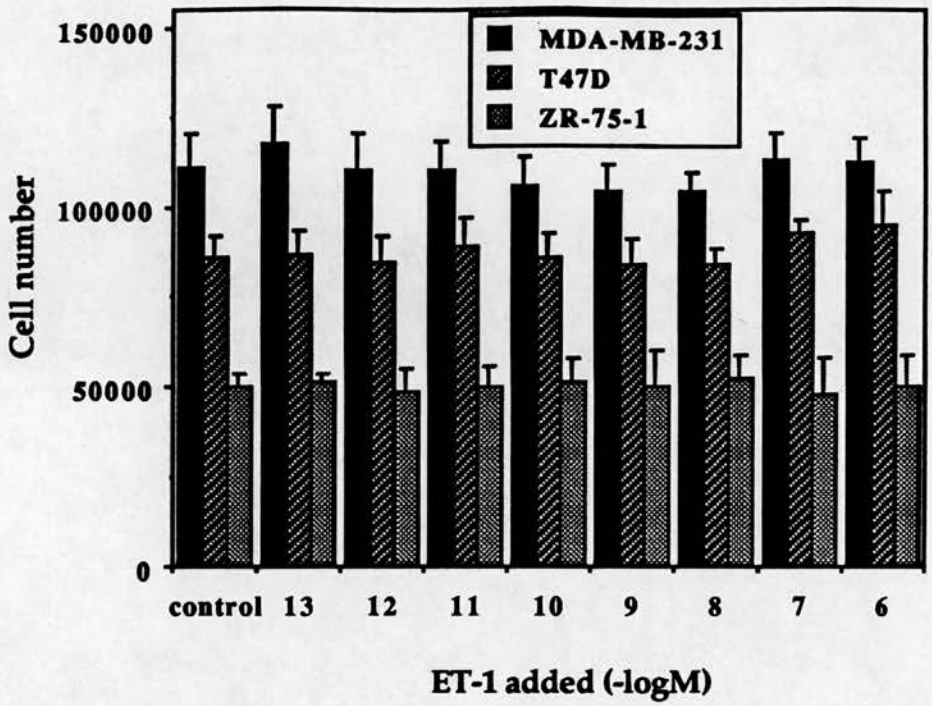


Figure 6.7: Effects of the exogenous addition of ET-1 on MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

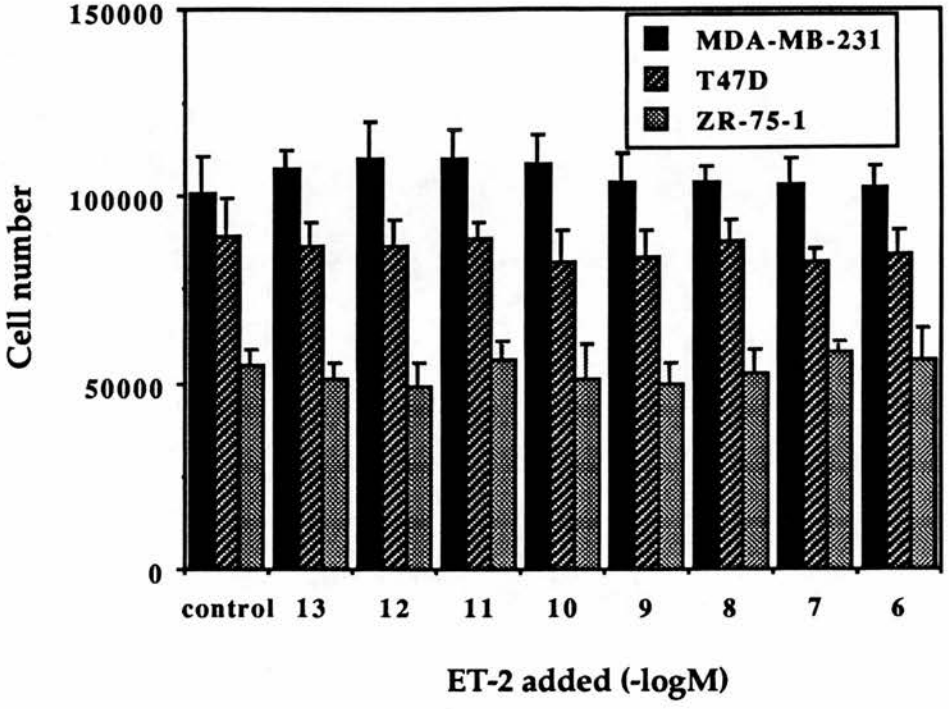


Figure 6.8: Effects of the exogenous addition of ET-1 on MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

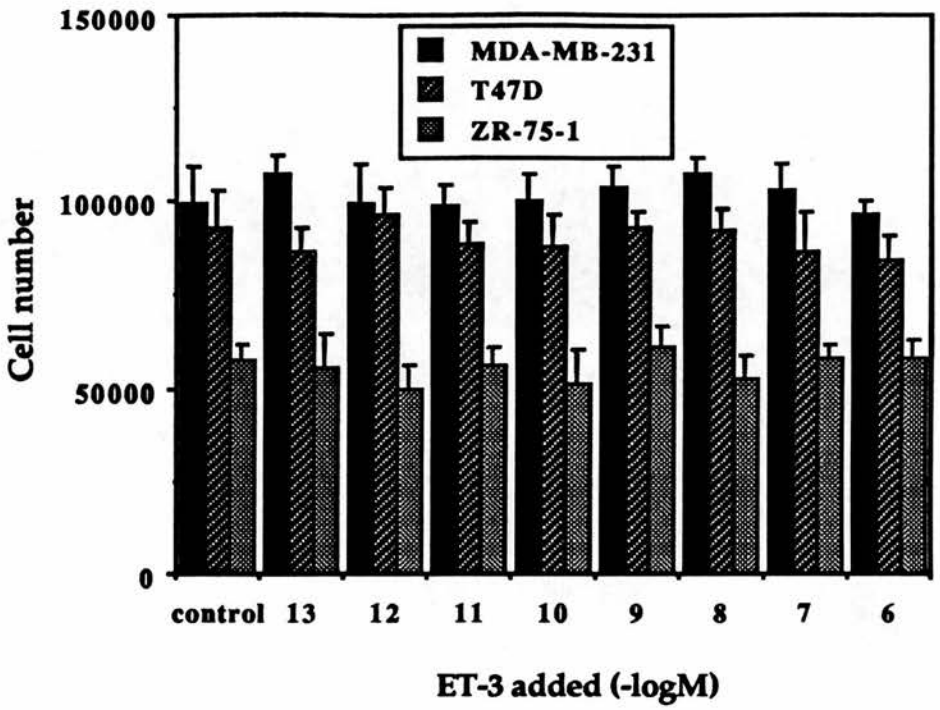


Figure 6.9: Effects of the exogenous addition of ET-1 on MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

6.4 Effect of the exogenous ET addition on growth of human breast fibroblasts

The growth stimulatory effects of ETs on human breast fibroblasts were investigated with the exogenous addition of ETs to BRF1 and BRF2 human breast fibroblast cell lines. Values shown in figures and tables were obtained from a representative of 3 experiments performed on separate occasions.

(i) Effects of ET-1

The effects of ET-1 addition at concentrations between 10^{-13} and 10^{-6}M on the growth of BRF1 and BRF2 breast fibroblasts are shown in figure 6.10.

In BRF1 cells, addition of ET-1 resulted in a stimulation of growth at concentrations between 10^{-12} and 10^{-8}M. This stimulation followed the same "bell-shaped" pattern, seen with the ovarian cancer cells, with increasing levels of stimulation at 10^{-12} and 10^{-11} of ET-1, maximum effects at 10^{-10}M (145% control cell number) and lower effects at 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M. All the concentrations of ET-1 mentioned above produced statistically significant effects on growth apart from 10^{-7}M.

In BRF2 cells, stimulation of growth as a result of the addition of exogenous ET-1 was seen only at concentrations between 10^{-10}M and 10^{-8}M with maximum stimulatory effect seen at 10^{-10}M (152% compared to controls) (figure 6.10). At concentrations between 10^{-13}M to 10^{-11}M and 10^{-8} to 10^{-6}M no stimulatory effects were detected.

Table 6.7 summarises the results of ET-1 addition on the growth of BRF1 and BRF2 fibroblasts from a representative of three experiments expressed as % control cell number

(ii) Effects of ET-2

Results of the addition of ET-2 to BRF1 and BRF2 cells in terms of growth are shown in figure 6.1. In the case of BRF1 cells, ET-2 addition between 10^{-13} and 10^{-10}M resulted in increasing stimulation of growth. Maximum growth stimulation (161% control growth) was detected with the addition of 10^{-10}M of exogenous ET-2.

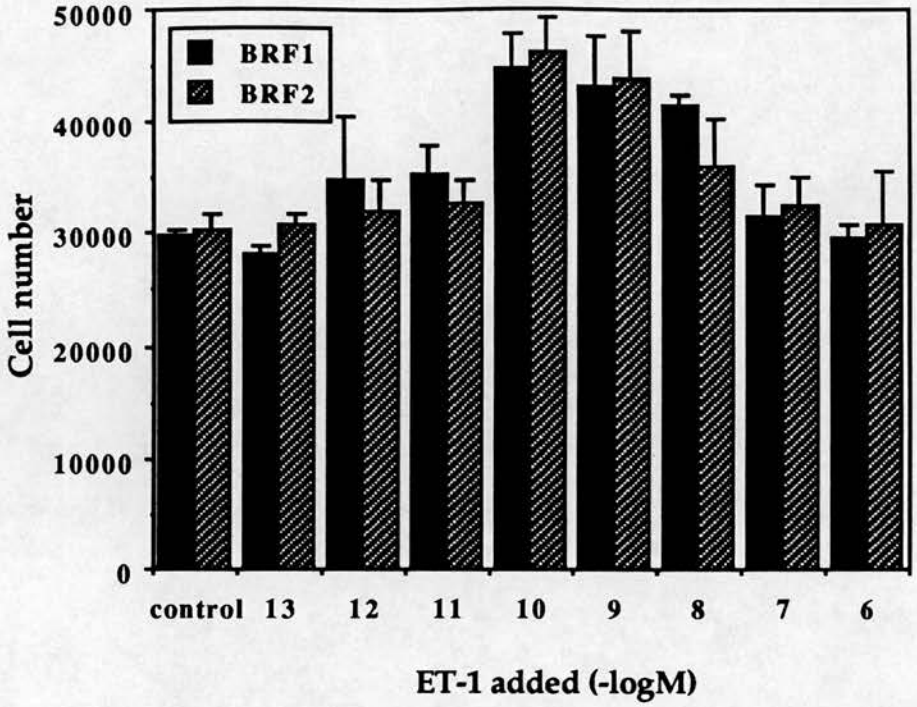


Figure 6.10: Effects of the exogenous addition of ET-1 on BRF1 and BRF2 breast fibroblast cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-1 on the growth of breast fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
BRF1	% control growth	95	* 115	* 120	* 145	* 132	* 127	103	98
	±se	2.9	23.6	17.3	12.7	18.5	3.7	11.7	5.26
BRF2	% control growth	101	105	108	* 152	* 145	* 119	107	101
	±se	9.7	7.2	10	14.2	13.7	8.6	7.7	7.6

Table 6.7. The effects of exogenous endothelin-1, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of BRF1 and BRF2 breast fibroblast cell lines expressed as % control cell number (2.9x104±496 and 3.0x104±2147 for BRF1 and BRF2 respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

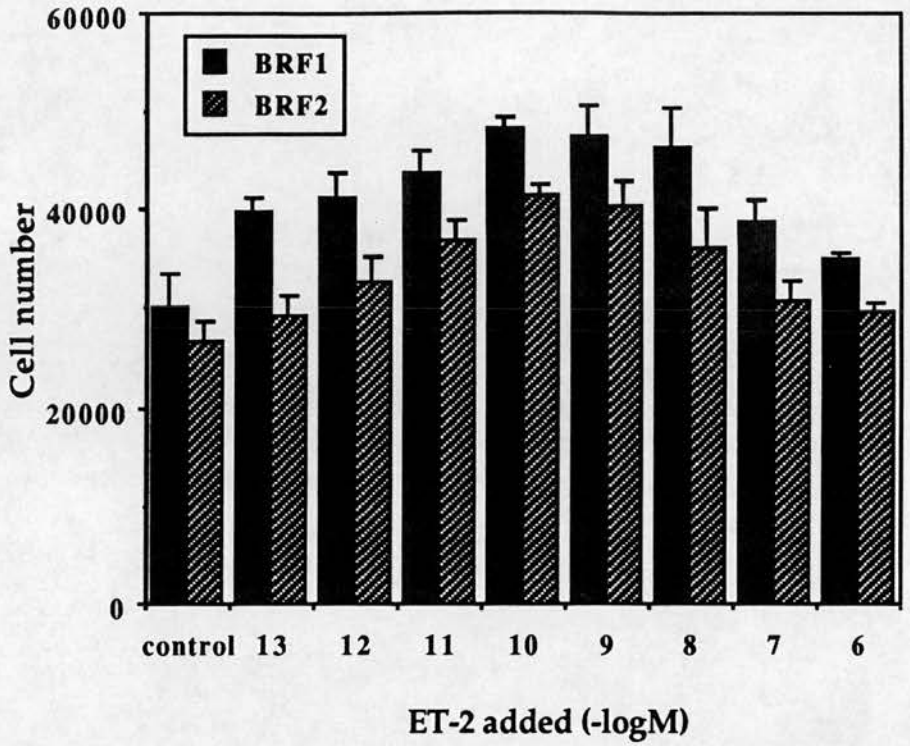


Figure 6.11: Effects of the exogenous addition of ET-2 on BRF1 and BRF2 breast fibroblast cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-2 on the growth of breast fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
BRF1	% control growth	* 133	* 137	* 146	* 161	* 158	* 154	* 129	* 116
	±se	4.3	8.3	7.2	3.2	11	12.9	7.2	2.1
BRF2	% control growth	* 109	* 122	* 137	* 154	* 151	* 134	* 115	* 111
	±se	6.9	9.7	7.5	4.8	9.2	6.2	7	2.9

Table 6.8: The effects of exogenous endothelin-2, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of BRF1 and BRF2 breast fibroblast cell lines expressed as % control cell number ($3.0x10^4$±3314 and $2.7x10^4$±2159 for BRF1 and BRF2 respectively). Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

At concentrations between 10^{-9} and 10^{-6}M the effects were decreasing.

In BRF2 cells, increasing stimulatory effects were detected at concentrations of 10^{-12} and 10^{-11} M with the maximum effects seen, as in BRF1 cells, at 10^{-10}M (154% control growth) of added ET-2 and lower effects at 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6}M.

Table 6.8 shows the effects of stimulation of growth (as % control cell number) for all the concentrations of ET-2 in BRF1 and BRF2 cells.

(iii) Effects of ET-3

The effects of ET-3 addition to BRF1 and BRF2 cells are shown in figure 6.12.

In BRF1 cells a small, almost equal, stimulation of growth was observed with the addition of ET-3 across the range of concentrations (10^{-13} to 10^{-6} M). At concentrations between 10^{-13}M and 10^{-11}M, a decrease in the effect was observed. Maximum effect was observed at 10^{-10}M (119% of control growth) and at higher concentrations (10^{-9} to 10^{-6}M) the effects were decreased and comparable to each other.

In BRF2 breast fibroblasts, added concentrations of ET-3 between 10^{-12} to 10^{-7}M resulted in an increased cell growth. The levels of growth stimulation were lower than stimulation with ET-1 or ET-2 at the same concentrations and followed the same "bell-shaped" pattern increasing between 10^{-12}M to 10^{-9}M (maximum effect at 10-9M showing a 26% increase in control cell number) and dropping with the addition of 10^{-8} and 10^{-7}M of ET-3 added respectively.

Table 6.19 summarises the effects of ET-3 addition at concentrations between 10^{-13} and 10^{-6}M for both BRF1 and BRF2 cell lines.

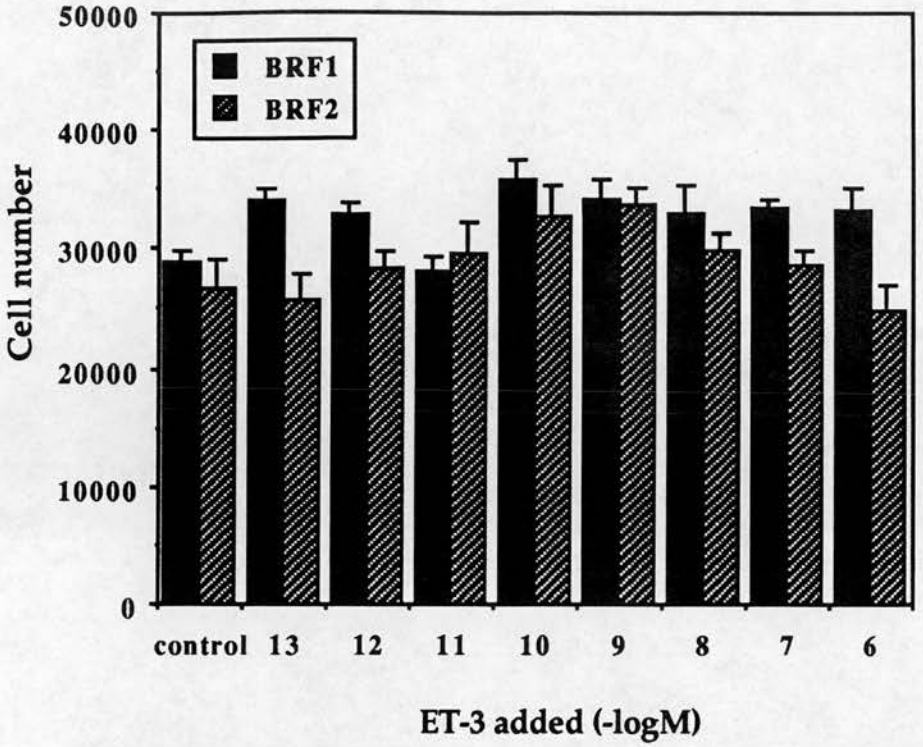


Figure 6.12: Effects of the exogenous addition of ET-3 on BRF1 and BRF2 breast fibroblast cell lines growing in 1% serum conditions for a 5 day period. Graph shows results from a representative of 3 experiments performed on separate occasions (bars: mean±se).

Effect of ET-3 on the growth of breast fibroblast cell lines									
Cell line	added ET (-logM)	13	12	11	10	9	8	7	6
BRF1	% control growth	* 114	* 111	104	* 119	* 114	* 111	* 112	* 112
	±se	2	2.1	3.2	4.5	4.2	5.8	2	5.4
BRF2	% control growth	97	* 107	* 111	* 123	* 126	* 121	* 107	94
	±se	10.1	4.8	9.5	9.4	4.9	4.9	4.6	4.5

Table 6.9. The effects of exogenous endothelin-3, added at concentrations of 10^{-13} to 10^{-6}M, on the growth of BRF1 and BRF2 breast fibroblast cell lines expressed as % control cell number ($3.9x10^4$±1899 and $2.6x10^4$±2875 for BRF1 and BRF2 cell lines respectively) . Each value represents mean ± se of three experiments performed on separate occasions (* values represent statistical significance, $p<0.05$).

6.5 Discussion

Evidence presented elsewhere in this thesis suggests that epithelial cancer cell lines but not fibroblasts produce significant amounts of ET-1-like material and that ovarian epithelial cancer and ovarian and breast fibroblast cell lines express specific ET-receptors. In this chapter we have shown that exogenous addition of ETs has been shown to affect the growth of these cell lines.

Thus, exogenous addition of ETs to PEO4 and PEO14 cells produced significant stimulatory effects on the growth of both ovarian carcinoma cell lines. The effect of exogenous ET-1 addition was concentration dependent with the maximum effects shown at 10^{-10}M for PEO4 and at 10^{-9}M for PEO14 cells. The minimum ET-1 concentration necessary to produce significant increase in cell number in both PEO4 and PEO14 was 10^{-12}M. This concentration is higher that the circulating ET-1 concentration in humans (10^{-13}M) (Huggins, 1993) but 100 times less than the concentration (10^{-10}M) reported to produce mitogenesis in fibroblasts and messangial cells (Huggins, 1993). Furthermore, exogenous addition of ET-1 between 10^{-10} and 10^{-7}M (maximum effect with 10^{-7}M) stimulated thymidine incorporation in SK-OV-3 and cell growth (in cell numbers) in OVCA 433 cells (Bagnato et al., 1995). In the present study, ET-2 also stimulated an increase in cell numbers in a concentration dependent manner but quantitative differences were evident between the cell lines, PEO14 cells appearing more sensitive. Thus, 10^{-8}M was necessary to stimulate maximum effects (20% increase in cell growth) in PEO4 cells compared to 10^{-9}M needed to produce maximum effects in PEO14 cells. The effects of ET-1 and ET-2 on both ovarian epithelial cancer cell lines show a biphasic nature with a smaller effect being seen at higher exogenous concentrations (10^{-7}M) than lower exogenous concentrations (10^{-9}M) of the peptide added to the cells. Such "bell shaped" effects on growth have been observed with the peptides and could be explained by the downregulation of endothelin receptors by the addition of high concentrations of ET-1. Indeed such effects have been described in

described in endothelial and smooth muscle cells which secrete ET-1-like material and also express endothelin receptors on their membranes (Clozel et al., 1993). Exogenous addition of ET-3 did not result in any significant mitogenic response in either of the ovarian cell lines and at any concentration (10^{-13} to 10^{-6}M) tested. These results on the effects of ETs on the growth PEO4 and PEO14 ovarian cancer cell lines support the observations of the binding experiments (chapter 5) which suggested that the cell lines express ET_A-R subtypes which are shown to have very low affinity for ET-3 (which would then have no effect on their growth), high affinity for ET-2 and highest of ET-1.

The exogenous addition of ETs to PEO12F, PEO14F and PEO27F ovarian fibroblasts produced significant stimulatory effects on the growth. The addition of ET-1 exerted mitogenic effects between concentrations of 10^{-13} to 10^{-6} (10^{-12} to 10^{-9} for PEO27F cells) with the maximum effect seen at 10^{-10}M in all 3 cell lines. ET-2 addition also had similar stimulatory effects at concentrations between 10^{-12} and 10^{-7} in all cell lines with maximum influences being seen at 10^{-9} M (PEO12F and PEO14F) and 10^{-10}M (PEO27F). In contrast to ovarian epithelial cancer cell lines, exogenous addition of ET-3 between 10^{-12} and 10^{-6}M had the same growth stimulatory effects in all 3 fibroblast cell lines as ET-1 and ET-2, maximum effects were observed at 10^{-9}M (PEO12F, PEO14F) and 10^{-8}M (PEO27F). The magnitudes of the effects seen with each endothelin were similar in all cell lines. These observations suggest that these effects were mediated at least in part through ET_B receptors although results from binding experiments showed the expression of both types of ET-Rs.

The addition of exogenous ETs to MDA-MB-231, T47D and ZR-75-1 cells did not have any effects on the growth of the three breast epithelial cancer cell lines in culture, a result is in accordance with the absence of ET receptors on the cells (chapter 5). Other studies have also reported the absence of ET receptors in breast epithelial and cancer cell lines (Baley et al., 1990).

Exogenous addition of ETs however had significant effects on the growth

of BRF1 and BRF2 breast fibroblast cell lines. Addition of ET-1 exerted a mitogenic effect on both cell lines at concentrations of 10^{-12} and 10^{-7}M with maximum stimulation observed at 10^{-10}M. Addition of ET-2 to BRF1 cells showed even greater effects than ET-1 at the same range of concentrations and with maximum effects at 10^{-10} and 10^{-9}M. In BRF2 cells, ET-2 had very similar effects to ET-1 with maximum growth stimulation at 10^{-10}M but smaller than the effect seen with ET-1. ET-3 addition also affected growth of the cells in both cell lines. In BRF1 cells, the effects were not as substantial as with ET-1 and ET-2 but were still significant between concentrations of 10^{-13} to 10^{-12}M and 10^{-10} to 10^{-6}M. Maximum effects were observed at 10^{-10}M. In BRF2 cells effects were observed between 10^{-11} and 10^{-7}M with maximum stimulation of growth at 10^{-10} and 10^{-9}M. The results suggested that BRF2 cells were more sensitive to ET-3 than BRF1 cells. As with ovarian fibroblast cell lines the above results would suggest that effects of ETs are mediated at least partially through ET_B-R subtypes. Results from binding experiments have suggested that these cell lines express both ET_A-R and ET_B-R subtypes (chapter 5) and it is thus possible that ETs exert their mitogenic effects through both receptor subtypes. Reports on breast fibroblasts have suggested the expression of ET receptors in these cells without presenting data regarding the type of receptors (Baley et al., 1990).

Chapter 7: Effects of endothelin receptor antagonists on the growth of ovarian and breast epithelial cancer cells and fibroblasts.

The effects of the three endothelin subtypes on the growth of different cell types were described in chapter 6. In order to help determine the specific ET receptor subtypes involved in the growth of ovarian and breast epithelial cancer cell lines and fibroblasts, two endothelin receptor specific antagonists (BQ123, ET_A-R antagonist) and (BQ788, ET_B-R antagonist) were used. To ascertain whether the ET-receptor antagonists exert any effects on the growth of the cell lines experiments using BQ123 and BQ788 in the culture of the cell lines in the absence of exogenous ET-1 were carried out. Such experiments would also demonstrate the ability of these antagonists to block possible effects on growth as a result of the endogenous production of endothelin (already demonstrated in chapter 4) from PEO4 and PEO14 ovarian epithelial cancer cells.

7.1 Ovarian epithelial cancer cells

7.1.1 PEO4 cells

The effects of the two endothelin receptor antagonists on the growth of PEO4 cells are illustrated in figure 7.1.

In this cell line, addition of the antagonists BQ123 or BQ788 at 100nM in the absence of exogenous ET-1 did not significantly affect the growth of the cells. Addition of 10^{-9}M ET-1 to the culture of PEO4 cells in the absence of any ET receptor antagonists resulted in a 50% increase in cell number compared to controls (150% control cell number). However, the addition of 100nM of BQ123 (ET_A-R antagonist) in the presence of 10^{-9}M ET-1 resulted in 90% inhibition of the ET-1 induced growth suggesting the expression of ET_A receptors in PEO4 cells and their involvement in the growth stimulation of the cells as a result of exogenous ET-1 addition. The addition of 100nM of BQ788 (ET_B-R antagonist) in the presence of 10^{-9} M ET-1, significantly blocked (60%) the ET-1-induced growth. Although the extent of this inhibition was not as substantial as that produced by the ET_A-R antagonist, it was still significant suggesting the presence of ET_B

receptors which partially mediate the ET-1 induced stimulation of growth in PEO4 cells.

7.1.2 PEO14 cells

The effects of the ET antagonists BQ123 and BQ788 on the growth of PEO14 cells in the presence or absence of exogenous ET-1 are described in figure 7.2.

In contrast with PEO4 cells, the addition of 100nM of BQ123 (ET_A-R antagonist) in the absence of exogenous ET-1, resulted in significant inhibition of control growth. Such effect suggests inhibition of an effect on the growth of PEO14 cells caused by endogenous ET-1. Addition of 100nM of BQ788 (ET_B-R antagonist) in the absence of exogenous ET-1 had no effect on the growth of PEO14 cells. Exogenous ET-1 at 10^{-9}M and in the absence of ET-R antagonists, stimulated growth to 170% compared to control cell number. However, 10^{-9}M ET-1 in the presence of 100nM BQ123 resulted in no stimulation of growth suggesting that the receptors present in PEO14 cells are of the ET_A-R type and mediate the growth stimulatory effects of ET-1. Addition of 10^{-9}M of ET-1 in the presence of 100nM of BQ788 antagonist resulted in no inhibition of growth compared to growth with ET-1 alone, suggesting that ET_B receptors are either not present, or do not mediate the growth stimulatory effects of ET-1 in PEO14 cells.

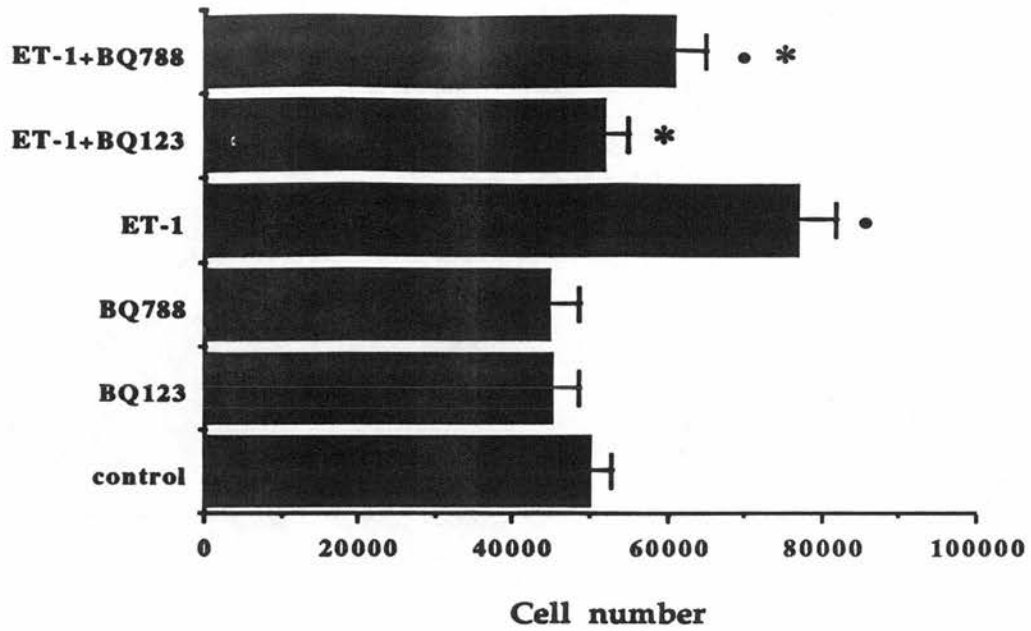


Figure 7.1: Growth of PEO4 ovarian epithelial cancer cells in culture, in the absence (control), or presence of: BQ123 ET_A-R antagonist (100nM), BQ788 ET_B-R antagonist (100nM), ET-1 (1nM), ET-1 (1nM) + BQ123 (100nM) and ET- 1(1nM) + BQ788 (100nM). Growth is shown as cell number at the end of a 5 days incubation and results represent mean values of three experiments performed on separate occasions (*) represents statistical significance ($p<0.05$) compared to ET-1 while (•) represents statistical significance ($p<0.05$) compared to control.

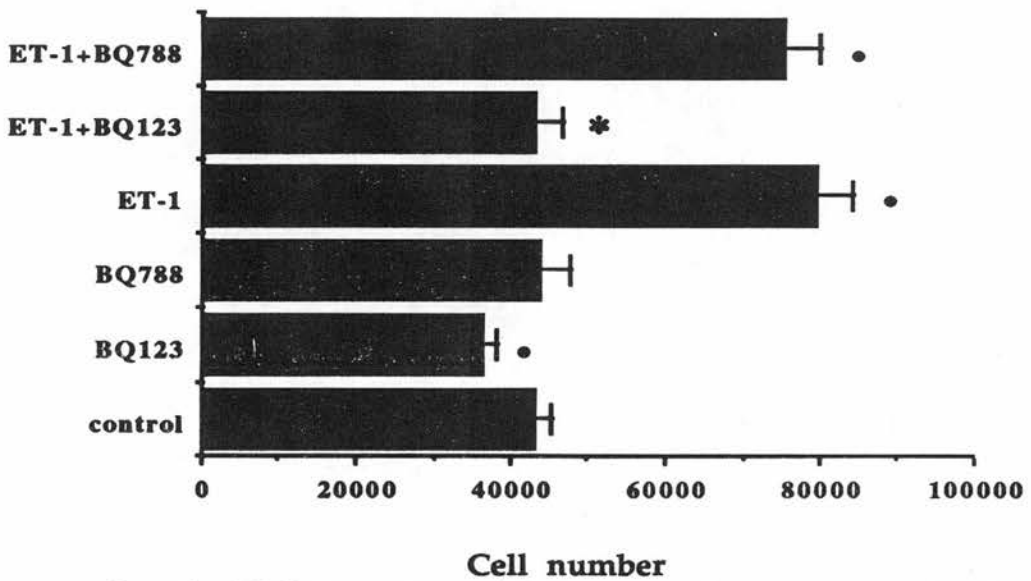


Figure 7.2: Growth of PEO14 ovarian epithelial cancer cells in culture, in the absence (control), or presence of: BQ123 ET_A-R antagonist (100nM), BQ788 ET_B-R antagonist (100nM), ET-1 (1nM), ET-1 (1nM) + BQ123 (100nM) and ET- 1(1nM) + BQ788 (100nM). Growth is shown as cell number at the end of a 5 days incubation and results represent mean values of three experiments performed on separate occasions (*) represents statistical significance ($p<0.05$) compared to ET-1 while (•) represents statistical significance ($p<0.05$) compared to control.

7.2 Ovarian fibroblasts

PEO12F ovarian fibroblasts were used in order to detect the effects of ET-R antagonists on the growth of ovarian fibroblasts in the presence or absence of exogenous ET-1 (figure 7.3).

Neither of the two ET-R antagonists had any effect on the growth of PEO12F cells in the absence of exogenous ET-1 while 10^{-9}M of ET-1 alone, stimulated the growth of the cells by 40% relative to the control cell number. The addition of 100nM of BQ123 (ET_A-R antagonist) in the presence of 10^{-9}M, partially blocked the growth stimulatory effect of ET-1 resulting in an increase of 16% of cell number compared to control. Such an effect suggested the expression of ET_A-R subtypes in PEO12F cells and the involvement of such receptors in the stimulation of growth of PEO12F cells by ET-1. The addition of 100nM of BQ788 ET_B-R antagonist to PEO12F cells in the presence of ET-1 also resulted in partial blockage of the stimulation seen with 10^{-9}M ET-1 in the absence of antagonists, showing an increase of 15% compared to control cell number. These results suggested that both types of ET receptors are expressed in ovarian fibroblasts and that they mediate the growth stimulatory effects of ET-1.

7.3 Breast fibroblasts

To identify the receptor subtype(s) mediating the growth stimulatory effects of exogenous ET-1 in breast fibroblasts in culture, the effects of the two ET-R antagonists BQ123 and BQ788 were investigated in BRF1 breast fibroblast cell line (figure 7.4). Incubation of BRF1 cells with 100nM of either BQ123 or BQ788 antagonist had no significant effect on the growth of the cells compared to controls. Incubation of BRF1 cells with 10^{-9}M of ET-1 in the absence of receptor antagonists resulted in 170% control cell number while 100nM of BQ123 in the presence of 10^{-9}M of ET-1 resulted in a reduced effect of ET-1 (125% of control cell number) on the growth of the same cells. Similar results were seen after the incubation of cells with 100nM of BQ788 and 10^{-9}M of ET-1 which also reduced the growth stimulatory effect of ET-1 to 113% control cell number. Finally incubation

of BRF1 cells with 10^{-9}M of ET-1 and both receptor antagonists (100nM of each) completely blocked the effect of ET-1 suggesting, as with PEO12F ovarian fibroblasts, the involvement of both receptor subtypes in mediating the growth stimulatory effects of ET-1.

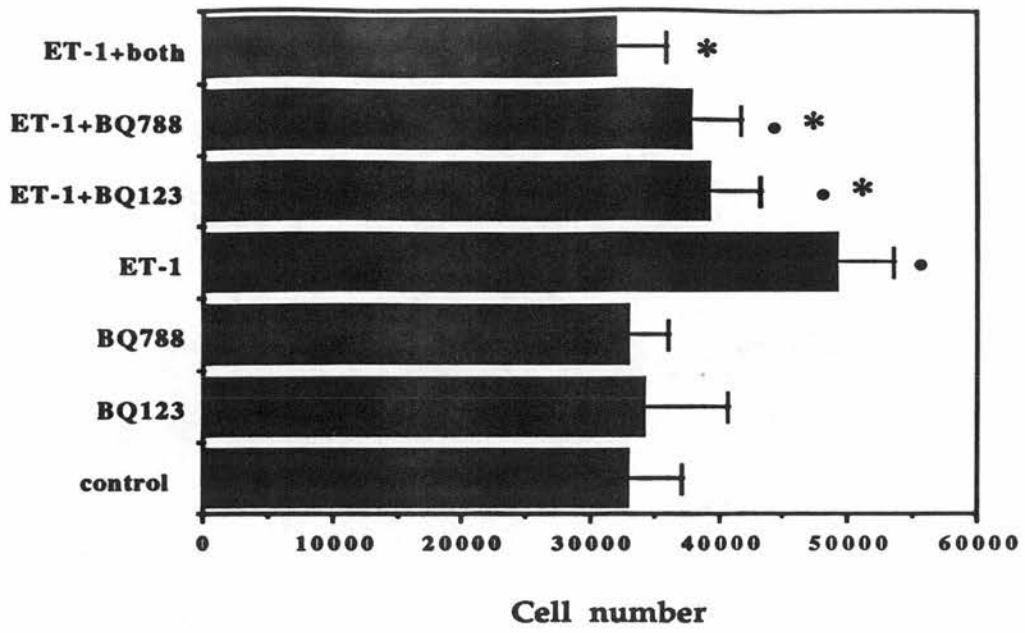


Figure 7.3: Growth of PEO12F ovarian fibroblast cells in culture, in the absence (control), or presence of: BQ123 ET_A-R antagonist (100nM), BQ788 ET_B-R antagonist (100nM), ET-1 (1nM), ET-1 (1nM) + BQ123 (100nM), ET-1 (1nM) + BQ123 (100nM) and ET-1 (1nM)+BQ123 (100nM) + BQ788 (100nM). Growth is shown as cell number at the end of a 5 days incubation and results represent mean values of three experiments performed on separate occasions ((*) represents statistical significance ($p<0.005$) compared to ET-1 while (•) represents statistical significance ($p<0.005$) compared to control.

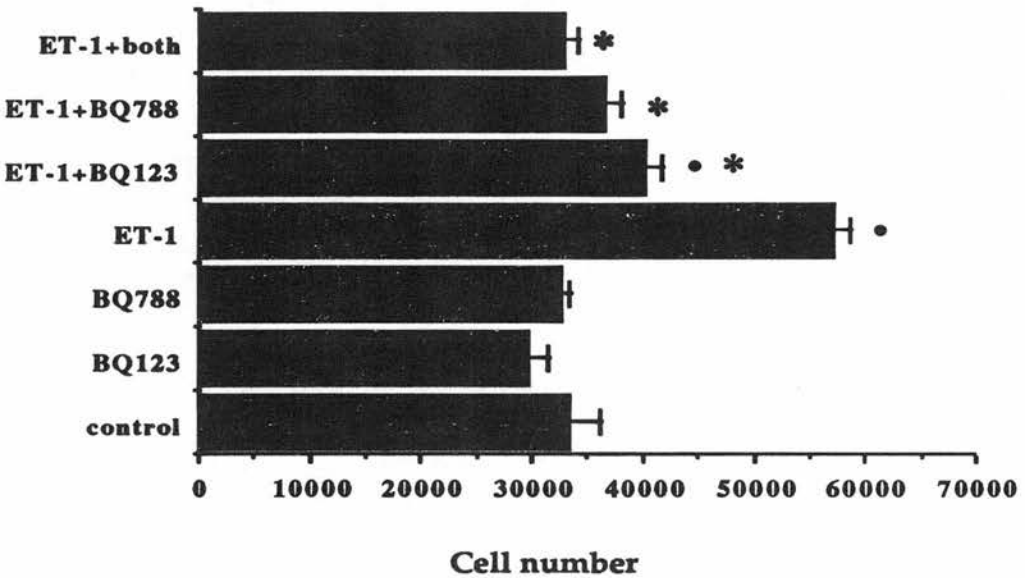


Figure 7.4: Growth of BRF1 breast fibroblast cells in culture, in the absence (control), or presence of: BQ123 ET_A-R antagonist (100nM), BQ788 ET_B-R antagonist (100nM), ET-1 (1nM), ET-1 (1nM) + BQ123 (100nM), ET-1 (1nM) + BQ123 (100nM) and ET-1 (1nM)+BQ123 (100nM) + BQ788 (100nM). Growth is shown as cell number at the end of a 5 days incubation and results represent mean values of three experiments performed on separate occasions (*) represents statistical significance ($p<0.005$) compared to ET-1 while (•) represents statistical significance ($p<0.005$) compared to control.

7.4 Discussion

The results presented in this chapter suggest that ET-receptor antagonists can be used in order to inhibit growth stimulatory effects (in terms of cell number) observed with the exogenous addition of ETs into the cultures of different cell lines, thus providing additional information regarding the subtypes of ET receptors mediating such effects.

A single report (Bagnato et al., 1995) using the same ET-R antagonists in order to block growth stimulatory effects of ET-1 on OVCA 433 cells showed that such effects were mediated by ET_A-R subtypes and could be blocked with the addition of BQ123 but not with the BQ788 antagonist.

In this study, results regarding the ovarian cancer cell lines, and the use of BQ123 receptor antagonist suggested that ET-1 exerted its mitogenic effects through ET_A-R in both cell lines. In the case of PEO4 cells only, a partial blockage of this effect was observed with the addition of BQ788 antagonist, suggesting the possible presence and involvement of ET_B receptors in the growth of these cells.

These results are in accordance with previous reports (Bagnato et al., 1995) and are consistent with the observations of the ligand binding experiments (chapter 5) which suggested the expression of ET_A receptors in both cell lines and the possibility of the presence of a small number of ET_B receptors in PEO4 cells.

Results from PEO12F ovarian fibroblasts have suggested that both receptor antagonists could partially block the growth stimulatory effect exerted on these cell lines by ET-1 thus suggesting the involvement of both ET_A-R and ET_B-R in the growth control of these fibroblasts. The consistent (non-significant) larger effect produced with the addition of BQ788 could account for differences in the number of the two receptor types expressed in PEO12F cells but could also account for the different degree of involvement of each receptor subtype in the growth of these cells.

In BRF1 breast fibroblasts the use of either BQ123 or BQ788 antagonists specifically blocked the effect of ET-1 on growth suggesting that both

receptor subtypes mediate such effects. The same (consistent but not statistically significant) observation, as in PEO12F cells, was made in BRF1 fibroblasts, in which BQ788 antagonist blocked the effect of ET-1 more effectively than BQ123 antagonist. The expression and involvement of the ET_B receptor subtype on the growth of ovarian and breast fibroblasts cells could also explain the mitogenic effects observed with the addition of ET-3 in the culture of these cells as suggested in chapter 6.

In conclusion, the growth stimulatory effects observed with the addition of ET-1 in PEO4 and PEO14 ovarian cancer cells in culture are consistent with mediation by the ET_A-R subtype but in the case of PEO4 cells with the ET_B-R subtype also. In ovarian and breast fibroblasts similar growth stimulatory effects of ET-1 could be mediated by both receptor subtypes.

Chapter 8: Co-culture experiments

To determine the effects of paracrine influences on the growth of epithelial and fibroblast cell types in ovarian and breast cancer, co-culture experiments were designed using tissue culture inserts in 24 well plates as shown in figure 8.1.

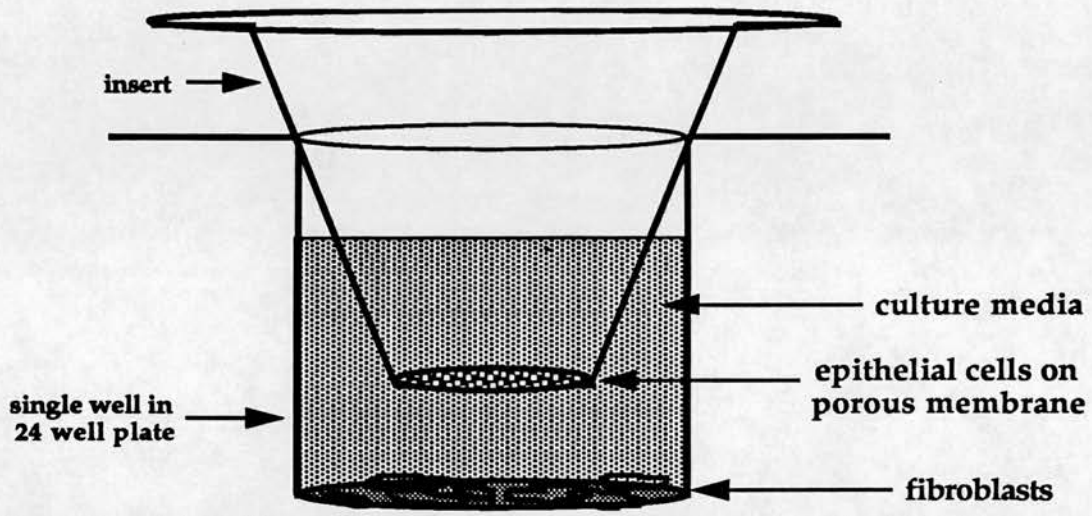


Figure 8.1: Schematic representation of the co-culture experiments. Fibroblasts were seeded at the bottom of 24 well plates and epithelial cells at the bottom of well inserts placed in separate wells and left for 24h in serum containing conditions (10% for PEO14, T47D and 15% for PEO12F and BRF1 cell lines). The inserts were then placed on top of the wells containing the fibroblasts and cultured together for 5 days in 1% serum conditions. The porous membrane at the bottom of the inserts allowed exchange of factors between the two cell types. At the end of the 5 day period inserts were separated, and cell counts from both populations were calculated using a Coulter counter.

The involvement of possible endogenous secretion of ETs from either epithelial and fibroblast cells and their effects on the growth of both were determined in the absence or presence of ET receptor antagonists. Epithelial cells and fibroblasts were seeded separately from each other in serum (10% and 15% respectively) containing media and then co-cultured in restricted serum conditions (1%). At the end of a 5 day incubation period, cells were trypsinised and cell numbers were determined by coulter counter. Results (in cell number) of each of the two cell types in culture in the absence or presence of ET-receptor antagonists were compared to results of epithelial and fibroblast cells growing separately.

8.1 Co-culture of ovarian epithelial cancer cells and fibroblasts

For the co-culture experiments with ovarian cells, PEO14 epithelial and PEO12F fibroblastic cell lines were used. The results of these experiments

are summarised in figures, 8.2 and 8.3 and are discussed in detail below.

After a 5 days incubation period, PEO14 cells growing in co-culture with PEO12F ovarian fibroblasts reached a significantly higher cell number (35% increase) compared to PEO14 cells growing separately. Similarly to the epithelial cells, the number of PEO12F cells in co-culture with PEO14 cells was significantly greater (37% increase) than that of PEO12F fibroblasts growing alone, suggesting functioning paracrine systems affecting the growth of both epithelial and fibroblast cell types.

With PEO14 cells, BQ123 significantly reduced (13%) the cell number compared to co-culture with no antagonist. However, the cell number of PEO14 cells in the presence of BQ123 remained significantly higher (17%) than that of PEO14 cells growing alone, suggesting the involvement of ETs and ET_A-R on the growth of these cells. Addition of the BQ788 ET_B-R antagonist into the co-culture of PEO14 resulted in no significant difference in their growth compared to controls.

The addition of BQ123 to PEO12F fibroblasts in co-culture significantly reduced (14%) their growth compared to PEO12F cell number in the absence of the antagonist. Cell number of PEO12F cells in the presence of BQ123 was still significantly higher (18%) than that of cells growing in isolation. Additionally, the BQ788 ET_B-R antagonist significantly reduced (15%) the growth of PEO12F fibroblasts. However, as with BQ123 cells growing in the presence of BQ788 reached significantly higher numbers (18%) compared to fibroblasts growing in isolation.

Table 8.1 summarises the results and the statistical analysis from the experiments described above.

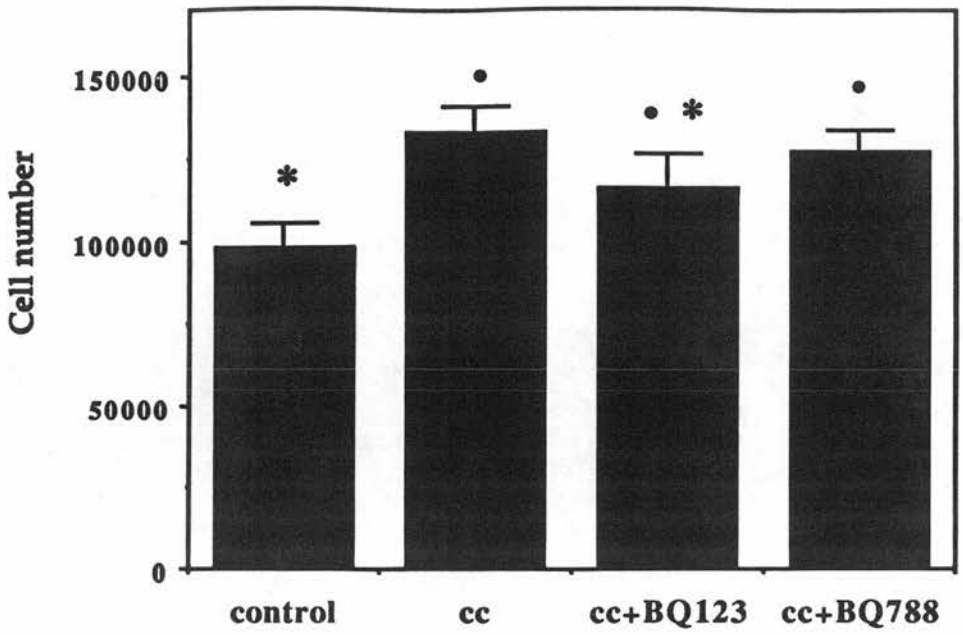


Figure 8.2: Growth (in cell number) of PEO14 ovarian epithelial cells growing in isolation (control) or in co-culture, in the absence (cc) or presence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists at the end of a 5 day incubation period. Asterisks (*) and points (•) represent values that reach statistically significant cell numbers compared to co-cultured and control cells respectively. Graphs shows mean values± standard error (se) of a representative of 3 experiments performed on separate occasions.

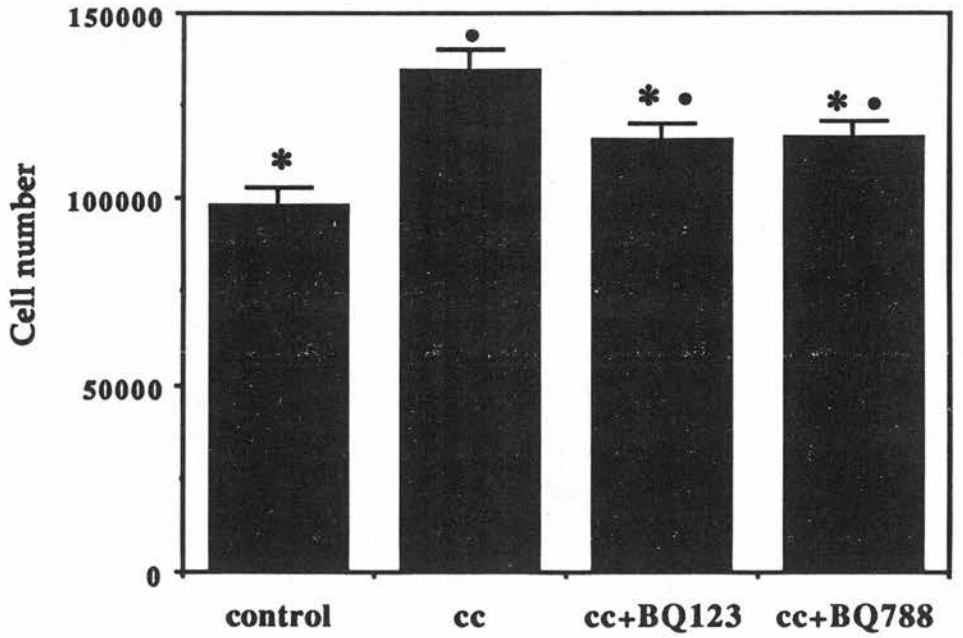


Figure 8.3: Growth (in cell number) of PEO12F ovarian fibroblasts growing in isolation (control) or in co-culture, in the absence (cc) or presence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists at the end of a 5 day incubation period. Asterisks (*) and points (•) represent values that reach statistically significant cell numbers compared to co-cultured and control cells respectively. Graphs shows mean values± standard error (se) of a representative of 3 experiments performed on separate occasions.

Cell line	conditions	Cell number ± se	p value compared to control	p value compared to co-culture
PEO14	control	98625 ± 3582	-	<0.0001 *
	co-cultured	132825 ± 4284	<0.0001 *	-
	cc+BQ123	115762 ± 5367	0.038 *	0.004 *
	cc+BQ788	127675 ± 2951	<0.0001 *	0.28
PEO12F	control	98088 ± 5162	-	0.003 *
	co-culture	134209 ± 5587	0.003 *	
	cc+BQ123	115850 ± 4277	0.04 *	0.043 *
	cc+BQ788	116538 ± 4365	0.038 *	0.046

Table 8.1: Table summarises numbers of PEO14 ovarian epithelial and PEO12F fibroblast cells growing in co-culture (cc) or in isolation, in the presence or absence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists and the statistical significance (p value) compared numbers of cells in isolation (control) or in co-culture (co-cultured). The asterisk (*) signifies values that reach statistical significance and results represent mean values±standard error (se) of a representative of three experiments performed on separate occasions.

8.2 Co-culture of breast epithelial cancer cells and fibroblasts

T47D breast epithelial cancer cells have been shown to secrete significant amounts of immunoreactive ET-1-like material as described in chapter 4 while their growth is not affected by exogenous addition of ET-1, 2 or 3 due to the lack of expression of ET-receptors as described in chapters 6 and 5 respectively. In contrast, BRF1 breast fibroblasts have been shown not to secrete ET-like peptide (chapter 4), to express both types of ET receptors (chapter 5) while their growth can be stimulated by all 3 ETs (chapter 6). T47D and BRF1 cell lines were used in order to study the paracrine role of ETs on the growth of breast epithelial and fibroblast cells in co-culture. The results are shown in figures 8.4 and 8.5 and are described in greater detail below.

T47D cells growing in co-culture with BRF1 fibroblasts grew to significantly higher (15%) numbers compared to T47D cells growing in isolation. Even greater stimulation (31%) of growth was detected in BRF1 cells in co-culture compared with fibroblasts growing separately, suggesting paracrine growth controls between the epithelial and fibroblast cells.

The addition of BQ123 (100nM) or BQ788 (100nM) antagonists into the co-culture of the cells had no effect on the growth of T47D epithelial cells.

However, the growth of BRF1 breast fibroblasts was inhibited by 12% (not quite statistically significant) and 16% with the addition of BQ123 and BQ788 antagonists (respectively) compared to cells growing in the absence of antagonists and in co-culture with T47D cells.

Table 8.2 describes the results and the statistical analysis of the experiments using breast epithelial and fibroblast cells in co-culture.

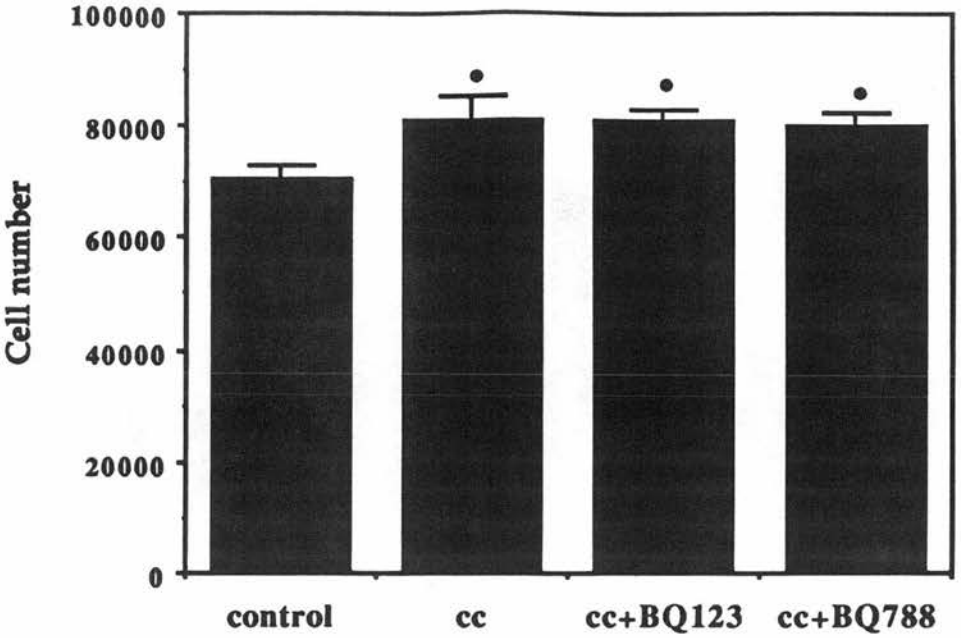


Figure 8.4: Growth (in cell number) of T47D breast epithelial cells growing in isolation (control) or in co-culture, in the absence (cc) or presence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists at the end of a 5 day incubation period. Asterisks (*) and points (•) represent values that reach statistically significant cell numbers compared to co-cultured and control cells respectively. Graphs shows mean values± standard error (se) of a representative of 3 experiments performed on separate occasions.

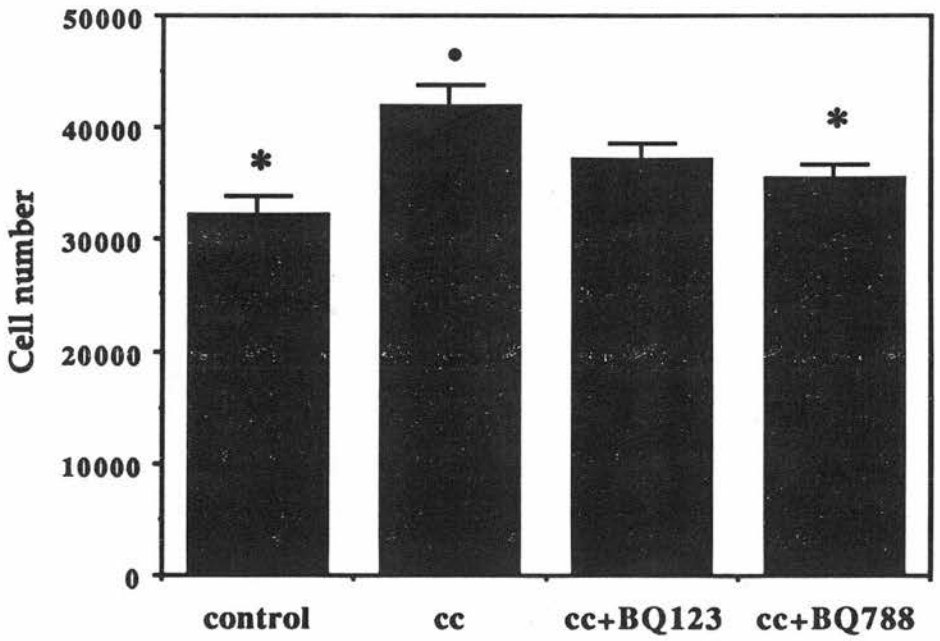


Figure 8.5: Growth (in cell number) of BRF1 breast fibroblasts growing in isolation (control) or in co-culture, in the absence (cc) or presence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists at the end of a 5 day incubation period. Asterisks (*) and points (•) represent values that reach statistically significant cell numbers compared to co-cultured and control cells respectively. Graphs shows mean values± standard error (se) of a representative of 3 experiments performed on separate occasions.

Cell line	conditions	Cell number ± se	p value compared to control	p value compared to co-culture
T47D	control	70458 ± 2437	-	0.007 *
	co-cultured	81154 ± 4372	0.007 *	-
	cc+BQ123	81254 ± 1774	0.022 *	0.5
	cc+BQ788	79987 ± 2293	0.029 *	0.5
BRF1	control	32157 ± 1797	-	0.007 *
	co-culture	42058 ± 1739	0.007 *	-
	cc+BQ123	37148 ± 1577	0.082	0.075
	cc+BQ788	35549 ± 1293	0.176	<0.024 *

Table 8.2: Table summarises numbers of T47D breast epithelial and BRF1 fibroblast cells growing in co-culture (cc) or in isolation, in the presence or absence of BQ123 (ETA-R) or BQ788 (ETB-R) antagonists and the statistical significance (p value) compared numbers of cells in isolation (control) or in co-culture (co-cultured). The asterisk (*) signifies values that reach statistical significance and results represent mean values±standard error (se) of a representative of 3 experiments performed on separate occasions.

8.3 Discussion:

As shown in the previous chapters, the ovarian (PEO4 and PEO14) and breast (MDA-MB-231, T47D and ZR-75-1) epithelial cancer cell lines, all secrete significant amounts of endothelin-1-like material. PEO4 and PEO14 cells, ovarian (PEO12F, PEO14F and PEO27F) and breast (BRF1 and BRF2) fibroblast cell lines all express endothelin receptors and respond to exogenous endothelins in terms of growth. Therefore, the involvement of endothelins in a paracrine regulation of growth is possible.

In this study, such involvement of endothelins was investigated with the addition of specific ET-receptor antagonists in the media of epithelial cancer cells and fibroblasts in co-culture. Numbers of cancer cells and fibroblasts in the presence or absence of ET-receptor antagonists in co-culture were compared to numbers of cells growing in isolation. Although the differences in cell number could be accounted for by the effects of other factors, the inclusion of ET-receptor antagonists enabled the detection of possible effects on growth due to endothelins.

Experiments involving PEO14 ovarian epithelial cells and PEO12F fibroblasts suggested that both cell types, grew significantly faster in co-culture than when growing separately. These results illustrated the influence of factors released, acting in a paracrine manner, on the growth of both cell types. The addition of BQ123 (ET_A-R) antagonist to the media of PEO14 and PEO12F cells in co-culture resulted in a partial but significant blockade of growth suggesting that ET_A receptors and therefore endothelins were involved in the paracrine regulation of growth. The fact that the antagonist blocked only a proportion of the effect suggested that other factors were also involved. The addition of BQ788 (ET_B-R) antagonist to the culture media of PEO12F cells resulted in a significant reduction of growth compared to cells in co-culture in the absence of the antagonist but had no effect on the growth of PEO14 cells suggesting the involvement of ET_B receptors in the regulation of growth in PEO12F fibroblasts but not in the PEO14 epithelial cancer cells.

In previous chapters it has been concluded that PEO14 cells produce

significant amounts of ET-1-like material and express at least one type of ET receptor (ET_A-R) while PEO12F fibroblasts do not produce any ET-1-like material but do express possibly both types of ET receptors(ET_A-R and ET_B-R). According to the results analysed above both cell types grow faster when in co-culture suggesting paracrine interactions which affect their growth. The use of ET-receptor antagonists in the culture media of both cell lines suggested that these paracrine interactions in addition to the autocrine interactions demonstrated in chapter 7 affecting cell growth do involve ET-1-like material secreted by PEO14 cells and ET receptors expressed in both PEO14 cells (ET_A-R) and PEO12F fibroblasts (ET_A-R and ET_B-R).

Co-culture experiments using T47D breast epithelial and BRF1 fibroblast cell lines also demonstrated the growth stimulatory effects exerted by factors acting in a paracrine fashion. Both cell lines grew faster when in co-culture than when growing in isolation although that difference was not significant in the case of T47D cells. The addition of BQ123 (ET_A-R) antagonist in the culture media of T47D cells in co-culture had no effect on their growth suggesting that ET_A receptors have no role in the growth of these cells. However, addition of BQ123 into the media of BRF1 cells resulted in a consistent although not quite statistically significant inhibition of growth suggesting a possible involvement of ET_A receptors in the growth of these cells.

Addition of BQ788 (ET_B-R) antagonists to the culture of T47D cells failed to exert any effect on growth, while the same antagonist had a significant inhibitory effect on the growth of BRF1 cells in co-culture.

The previous experiments demonstrated that T47D cells produce significant amounts of ET-1-like material (able to bind to both ET_A and ET_B receptors) while they do not express ET receptors while BRF1 cells do not produce any detectable amounts of ET-1-like material but seem to express both types of ET receptors. As shown in this chapter both cell lines grow faster in co-culture than in isolation suggesting paracrine interactions which in the case of T47D cells do not involve endothelins

since their growth is not affected by ET-R antagonists while in the case of BRF1 cells, such interactions involve ETs produced by T47D cells and acting through ET_B and possibly ET_A types of receptors.

Chapter 9: The use of antisense oligonucleotide technology to investigate the autocrine role of ETs in the growth of ovarian cancer cell lines

Specific "antisense"oligonucleotide sequences were designed to target the mRNAs of both endothelin receptors and were added into cultures of PEO4 and PEO14 cells in order to inhibit the effects of possible autocrine production of ETs on the growth of these cell lines. "Sense" and "random" oligonucleotide controls were also synthesised and their effects compared with the antisense sequence.

For both cell lines, the oligonucleotides were added at concentrations of 10 and 30μM (as described in the methods section). With PEO4 cells, as shown in figure 9.1, the addition of "antisense", "sense' or "random" oligonucleotides for either ET_A-R or ET_B-R had no effect on the growth of these cells. With PEO14 cells however (figure 9.2), the addition of "antisense" for ET_A-R produced significant inhibitions at both concentrations. Addition of "sense", and "random" had no effects at either 10 or 30μM suggesting that the effects were specific. The addition of ET_B-R "antisense" or "sense" produced no effects compared to control growth in this cell line.

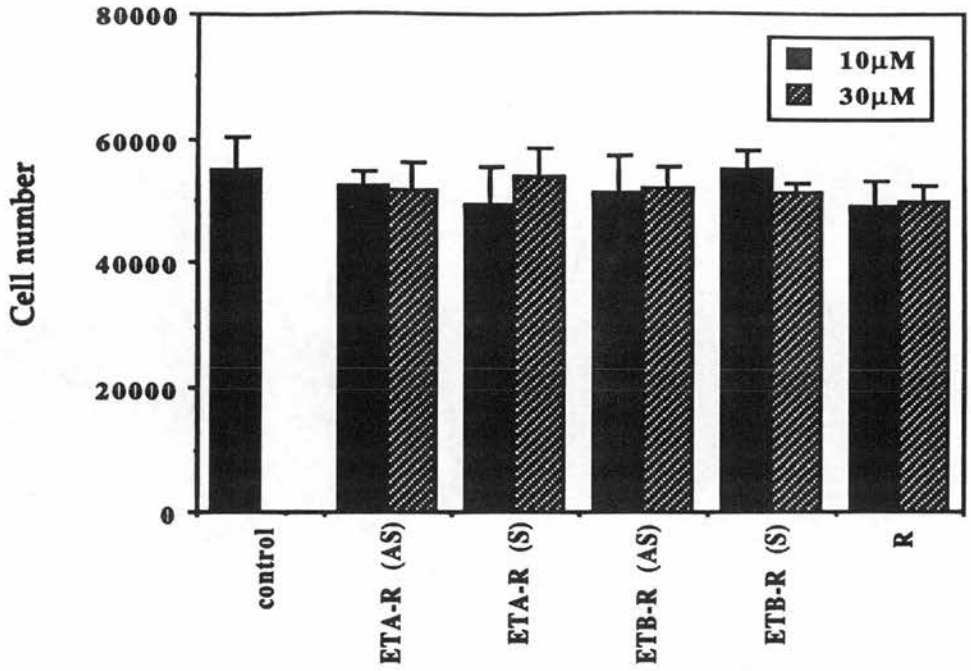


Figure 9.1: Effects of the use of "antisense" (AS), "sense" (S) and "random" (R) oligonucleotides for ET_A-R and ET_B-R at concentrations of 10 and 30μM on the growth of PEO4 cells after a 7 day incubation period in serum free media. Day zero counts for the controls were 30128±519 cells. Results shown are mean±se values from a representative of 3 experiments performed on separate occasions.

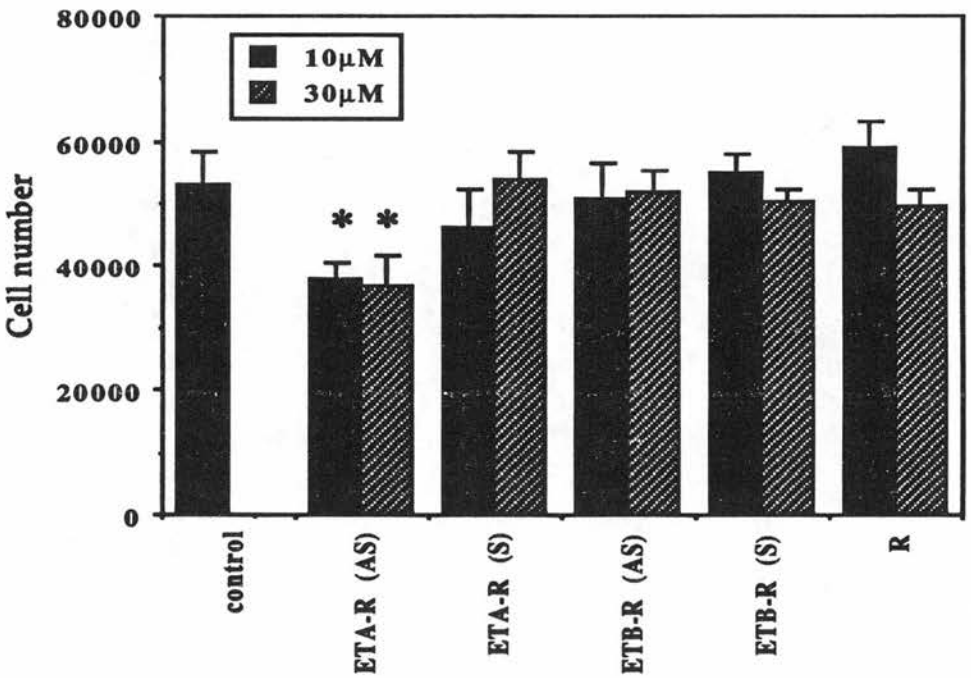


Figure 9.2: Effects of the use of "antisense" (AS), "sense" (S) and "random" (R) oligonucleotides for ET_A-R and ET_B-R at concentrations of 10 and 30μM on the growth of PEO14 cells after a 7 day incubation period in serum free media. Asterisk (*) represents statistical significance ($p<0.05$) compared to control values. Day zero counts for the controls were 29758±875 cells. Results shown are mean±se values from a representative of 3 experiments performed on separate occasions.

9.2 Discussion

In the previous chapters, it has been shown that PEO4 and PEO14 cells secrete significant amounts of ET-1-like material, that they express ET receptors, are growth responsive to exogenous ET addition and that this effect can be blocked with the addition of specific receptor antagonists. In this chapter we have investigated the possible effect of the endogenous ET on the growth of the same cell lines by studying the impact of "antisense" oligonucleotides for both ET receptors in order to block the production of ET receptors. The use of antisense oligonucleotides against the ET-receptors and not against ET-peptides was preferred. The reasoning behind such decision was the problems of different affinities of the two receptor types for the ET-peptides, and the fact that by using the antagonists selected, more information would be revealed regarding the receptor types involved and results would be directly comparable with the results of the co-culture experiments. Results shown in this chapter represent the first data of the use of antisense technology for endothelin receptors and only the second set of data of antisense technology in ovarian cancer. In the previous study, antisense oligonucleotides were used against the IGF receptor (Resnicoff et al., 1993).

The results showed no effects on the growth of PEO4 cells suggesting that either the amount of ET secreted does not have any effect on their growth or that the number of receptors expressed is low and therefore the receptor signalling is not sufficient, or that the effects of "antisense" were not sufficient to block the autocrine effect. However, previous experiments (chapter 6) have illustrated that exogenous addition of ETs can stimulate growth responses, suggesting that the number of receptors expressed is sufficient for the effect to be detected.

For PEO14 cells, it was shown that the use of "antisense" inhibition of the ET_A-R resulted in a 58-63% decrease in cell number suggesting the involvement of this receptor type in the autocrine regulation of growth from ETs. The growth of PEO14 cells was not affected by the addition of antisense for the ET_B-R, and these observations are consistent with

indications that ET_B receptors are not expressed in PEO14 cells. "Sense" and "random" oligonucleotides had no effect on the growth of the same cells suggesting that the effects seen with ET_A-R antisense were genuine and specific. Fully defined conditions serum-free culture conditions (HITS) were used so that the only source of endothelin was from the cells themselves.

Comparing RIA results for the two cell lines (chapter 4) PEO14 cells were shown to produce 10 times the amount of ET-1-like material compared to PEO4 cells and such difference might explain an increased dependancy for PEO14 on an ET/ET-R autocrine loop (dilutions of HITS radioimmunoassayed showed no measurable ET-1-like material).

In summary, the results presented above suggest that in PEO14 (but not PEO4) ovarian cancer cells an autocrine system of regulation of growth is functional, and mediated by the ET_A receptor expressed in these cells.

Chapter 10: Expression of ET peptides and receptors using the Reverse Transcriptase Polymerase Chain Reaction method

To help establish the isoform of endothelin (produced by the cell lines used for radioimmunoassay experiments (chapter 4), the type of ET-receptors, and to determine whether the same peptides are expressed in tumour samples as in model systems, the expression of the 3 endothelin peptides and the ET receptor types at the RNA level in ovarian and breast cancer cell lines, fibroblasts and primary tumour samples, was investigated using RT-PCR technology (primers sequences shown on page 62). Throughout the experiments described below, ß-actin was used as a positive control and negative controls constituted of RNA samples that were not taken through the RT step but were amplified for 30 cycles in a PCR reaction with the appropriate primers. Further controls were performed in some of the experiments using specific oligonucleotides designed for probe labelling (Southern blotting, as described on page 64 of the methods).

10.1 Expression of mRNA for ET peptides and receptors in ovarian and breast cancer cell lines

10.1.1 Expression of ET peptides and receptors in ovarian cancer cell lines

RNA preparations from PEO4 and PEO14 cell lines were used to investigate the expression of ET1, ET-2, ET-3 peptides and ET_A and ET_B receptors (lanes 1 and 2 for PEO4 and PEO14 respectively). Results (figure 10.1) suggested the presence of single mRNA bands for ET-1, ET-3, ET_A-R and ET_B-R at the predicted sizes (582, 493, 368 and 530bp respectively). ß-actin bands of the predicted size (262bp) were also seen in PEO4 and PEO14 cell lines. Negative controls (lane 3 for PEO4 in ET-1,2,3 and for PEO14 in ET-Rs and ß-actin) produced no bands. Southern blotting experiments, using ET-1 and ET_A-R oligonucleotides, showed that the relevant bands observed in PCR gel represent ET-1, and ET_A-R (figure 10.2).

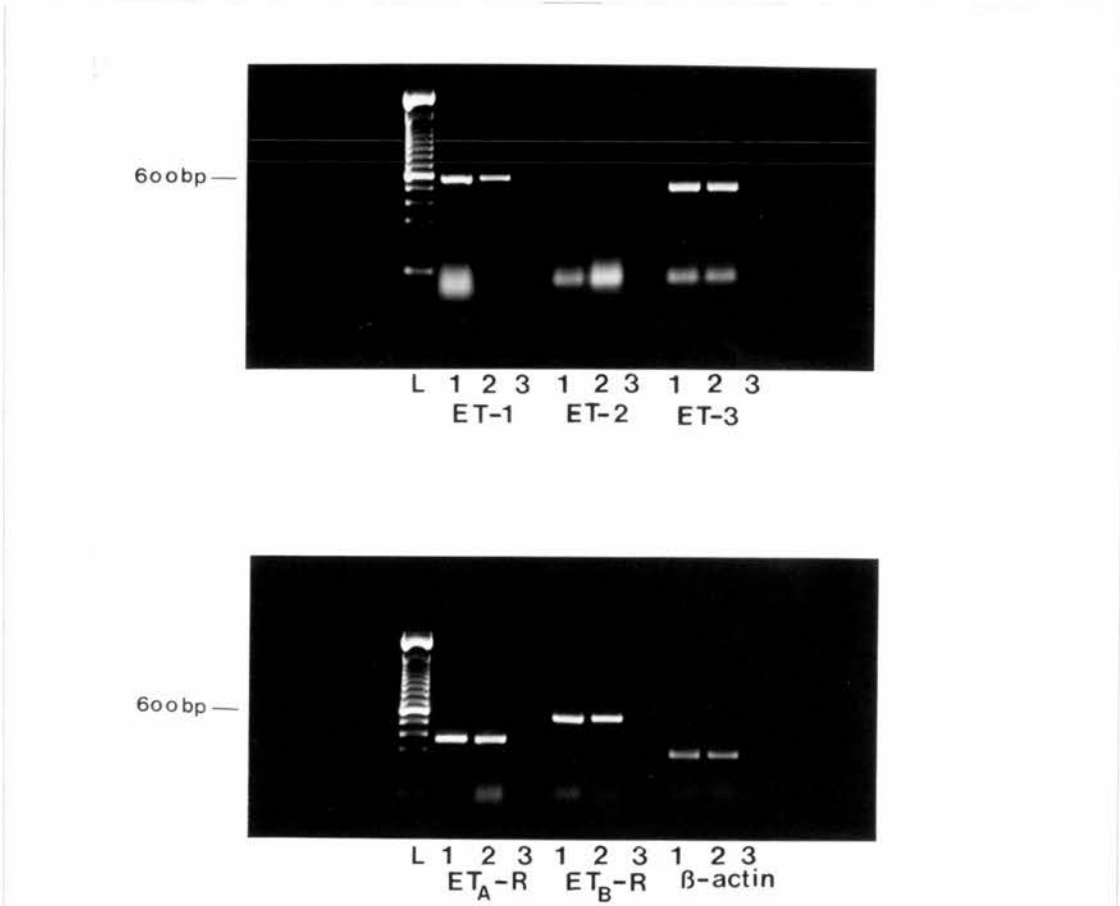
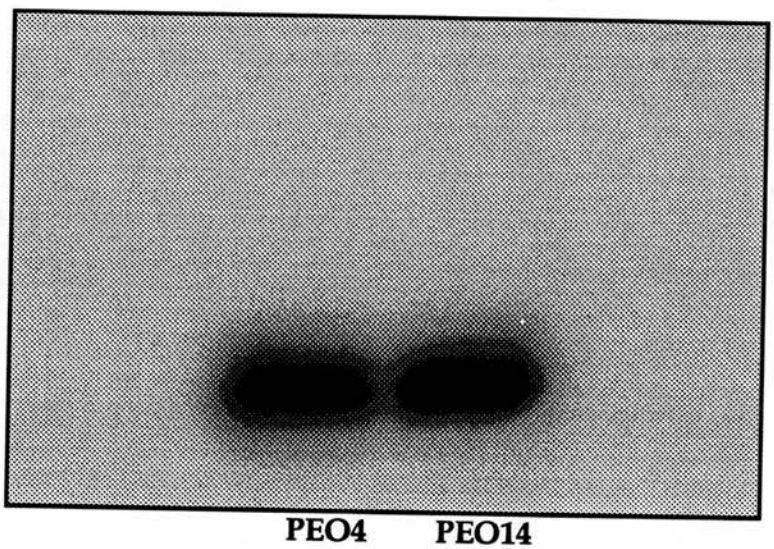
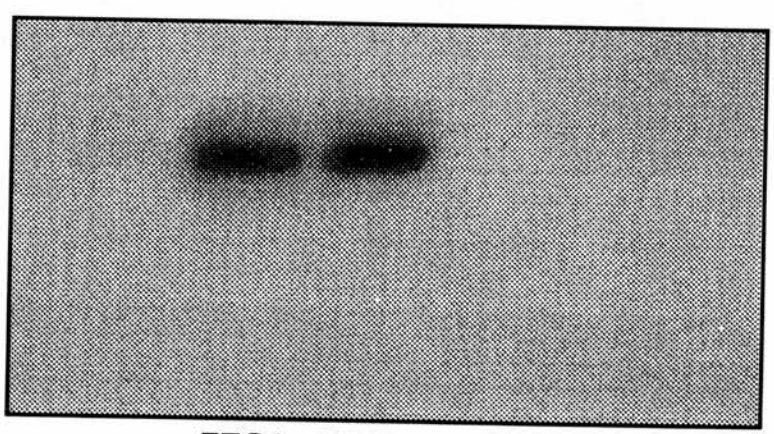


Figure 10.1: RT-PCR products of ET-1, ET-2, ET-3, ET_A-R, ET_B-R and ß-actin in PEO4 and PEO14 ovarian cancer cell lines. Lane L: 100bp DNA marker; lane 1: PEO4 cells; lane 2: PEO14 cells; lane 3: negative control samples (not through RT step). The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp, ET_A-R: 368bp, ET_B-R: 530bp, ß-actin: 262bp.



ET-1



ET_A-R

Figure 10.2: Southern blotting photographs of RNA bands in PEO4 and PEO14 cells detected with RT-PCR and labelled with specific oligonucleotides against ET-1 and ET_AR bands.

10.1.2 Expression of ET peptides and receptors in ovarian fibroblast cell lines

RNA from the ovarian fibroblast cell lines PEO9F, PEO12F, PEO14F and PEO27F were prepared and used for RT-PCR. Results (figure 10.3) suggested the expression of mRNA for ET-1, ET-3 (not in PEO27F) ET_A-R (not in PEO9F) and ET_B-R (lanes 1, 2, 3, 4, for PEO12F, PEO14F, PEO27F and PEO9F respectively) while no ET-2 mRNA was expressed. A ß-actin band was observed (262bp) in all cell lines while negative controls (PEO12F for ET-1 and ET-2; PEO14F for ET-3; PEO27F for ET_A-R and PEO9F for ET_B-R and ß-actin) showed no bands.

10.1.3 Expression of ET peptides and receptors in breast cancer cell lines

The expression of ET-1, 2, 3 and ET_A-R, ET_B-R mRNA in breast cancer cell lines was investigated using RNA preparations from four cell lines (MCF-7, MDA-MB-231, T47D and ZR-75-1). Results shown in figure 10.4 suggested the expression of mRNA in the form of single bands for ET-1 and ET-3 (at 582 and 493bp respectively) in all four cell lines (lanes 1, 2, 3, 4 for MCF-7, MDA-MB-231, T47D and ZR-75-1 cells respectively) apart from ET-3 in T47D cells, and the absence of mRNA for ET-2, ET_A and ET_B receptors in the same cell lines. Positive controls showed single band for ß-actin (at the predicted size of 262bp) in MDA-MB-231, T47D and ZR-75-1 (but not in MCF-7 cells although bands were observed for ETs). Figure 10.5 shows results for ß-actin expression in MDA-MB-231, T47D and ZR-75-1 cells from samples that have not been through the RT step (lanes 1, 2 and 3, respectively) and of identical samples that have been through the RT step (lanes 5, 6 and 7, respectively). A specific ß-actin band at 262bp was seen in all positive control and not in negative control samples.

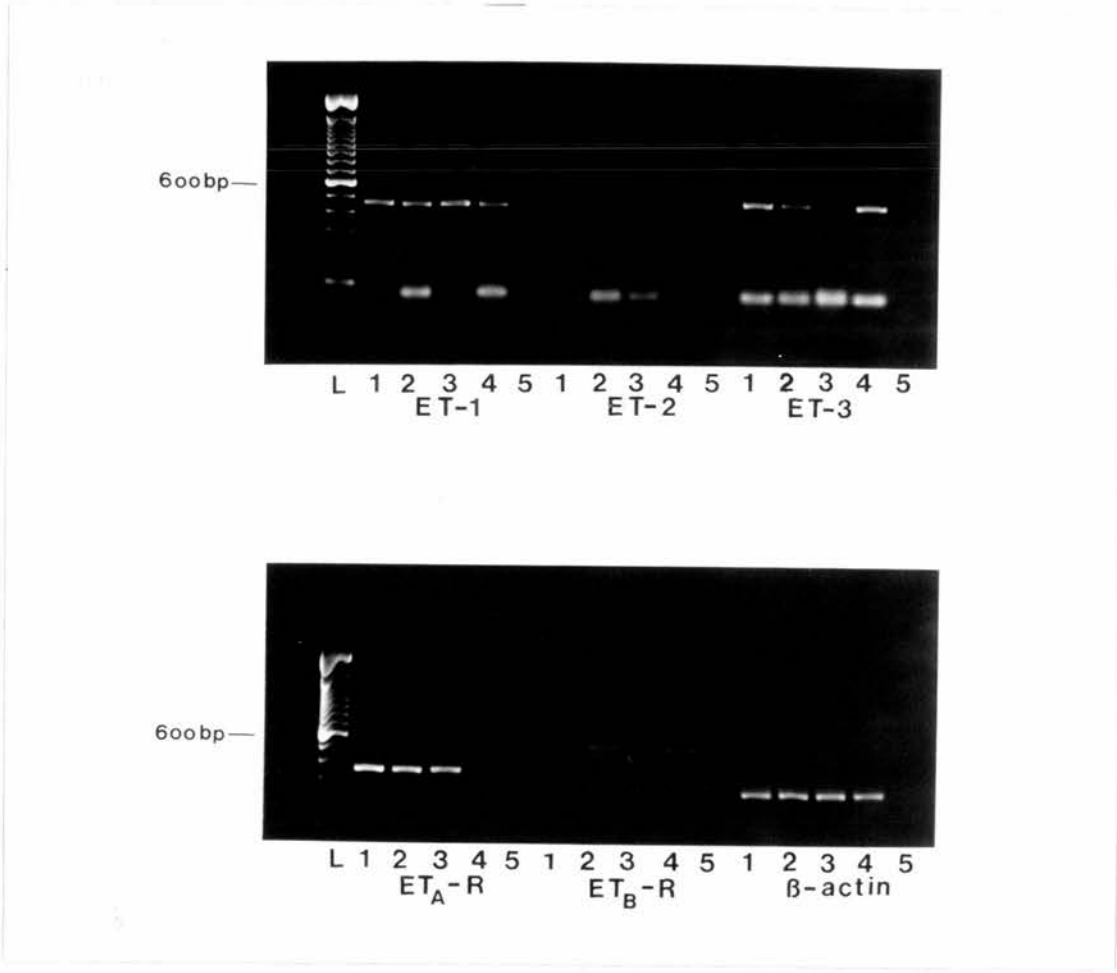


Figure 10.3: Photograph of an agarose gel of 30 cycle PCR products of PEO9F, PEO12F, PEO14F and PEO27F ovarian fibroblasts. Lane L: 100bp DNA marker; lane 1: PEO9F cells; lane 2: PEO12F cells; lane 3: PEO14F cells; lane 4: PEO27F cells; line 5: negative control samples (not through RT step). The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp, ET_A-R: 368bp, ET_B-R: 530bp, ß-actin: 262bp.

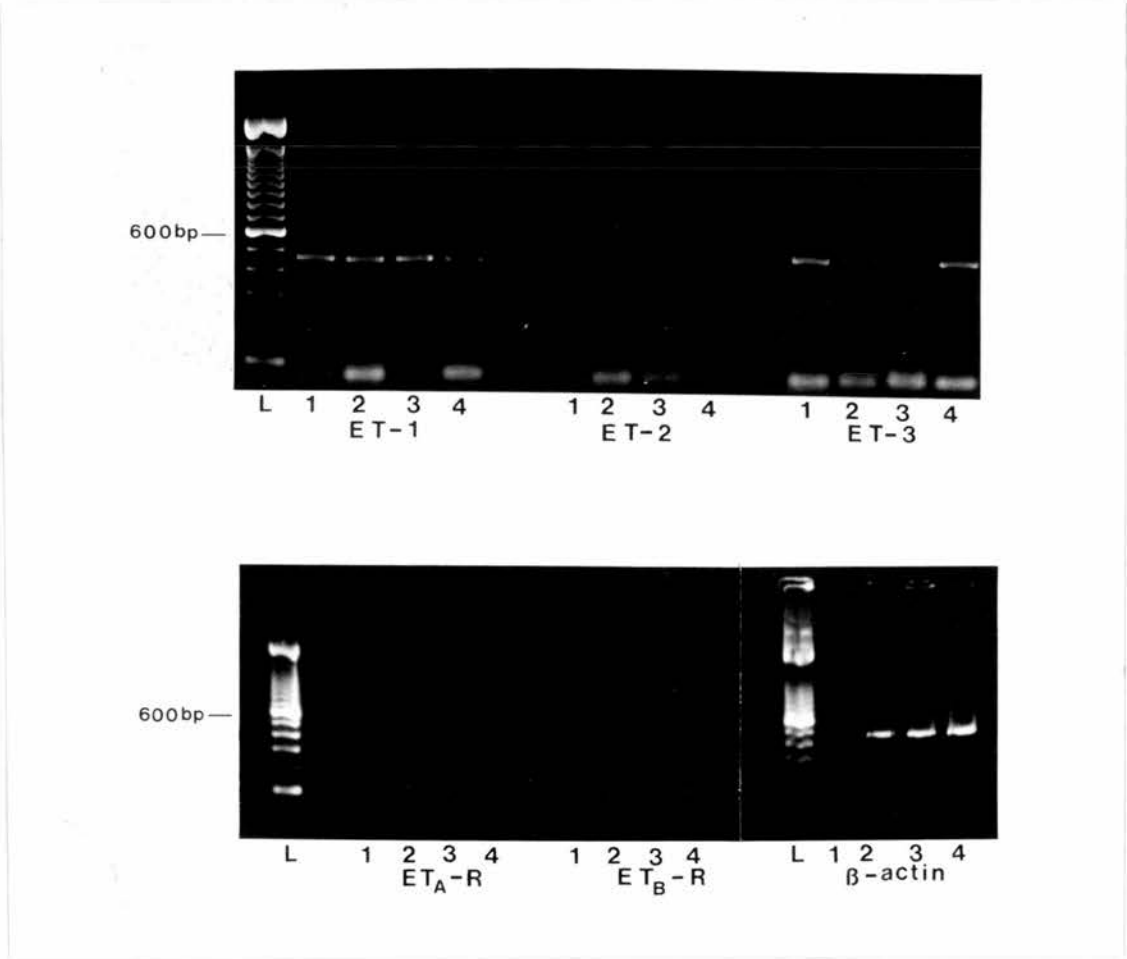


Figure 10.4: Photograph of an agarose gel of 30 cycle PCR products of MCF-7, MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines. Lane L: 100bp DNA marker; lane 1: MCF-7 cells; lane 2: MDA-MB-231 cells; lane 3: T47D cells; lane 4: ZR-75-1 cells. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp, ET_A-R: 368bp, ET_B-R: 530bp, ß-actin: 262bp.

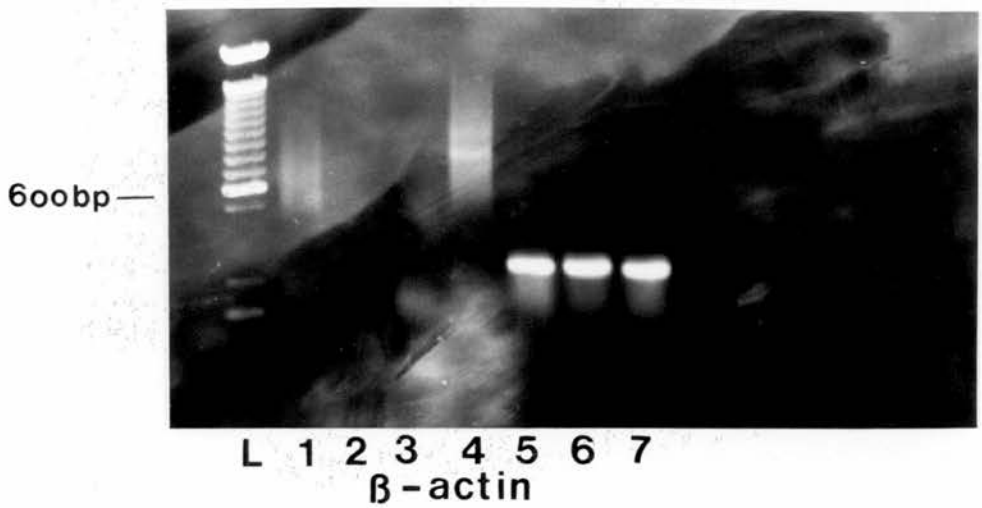


Figure 10.5: Photograph of an agarose gel of 30 cycle PCR products of MCF-7, MDA-MB-231, T47D and ZR-75-1 breast cancer cell lines. Lane L: 100bp DNA marker; lane 1: MDA-MB-231 cells; lane 2: T47D cells; lane 3: ZR-75-1 cells; lane 4: MDA-MB-231 (different sample); lane 5: MDA-MB-231 cells (no RT step); lane 6: T47D cells (no RT step); lane 7: ZR-75-1 cells (no RT step);. The predicted size for ß-actin: 262bp.

10.1.4 Expression of ET peptides and receptors in breast fibroblast cell lines

Figure 10.6 shows the expression of ET peptides and ET receptors in RNA preparations of BRF1 and BRF2 breast fibroblasts. From the three ET peptides only ET-1 mRNA was expressed as a single band at the predicted size of 582bp (lanes 2 and 3 for BRF1 and BRF2 respectively). Single bands for both ET_A (368bp) and ET_B (530bp) receptors were also detected in both cell lines (lanes 1 and 2 for BRF1 and BRF2 respectively). Negative controls were used in lanes 1 and 4 (for BRF1 and BRF2 respectively) for ET-1, ET-2 and ET-3 RNA samples only, which had not been through the RT step. No bands were detected in these lanes.

10.2 Expression of mRNA for ET peptides and receptors in ovarian and breast primary tumours

10.2.1 Ovarian primary tumours

Samples obtained from ten ovarian primary tumours were used to prepare RNA and to detect the expression of ET peptides and receptors. Results shown in figure 10.7 demonstrate the expression of ET-1 mRNA from all samples (ET-1 lanes, from 1 to 10), that of ET-2 from 6 out of 10 samples (ET-2 lanes; 2, 3, 4, 5, 8, 9) and ET-3 mRNA from 9 out of 10 samples (ET-3 lanes, from 1 to 10 apart from 2). Southern blotting experiments using the same gel and oligonucleotides for ET-1 and ET-3 both showed hybridisation of 10 bands for ET-1 and ET-3 at the exact positions shown on the PCR photograph (figure 10.8). Regarding the expression of mRNA of the two ET-receptor types, a single band was seen at 368bp for ET_A-R in all 10 samples (ET_A-R lanes, from 1 to 10), and for ET_B-R (at 530bp) in only 2 out of 10 samples (lanes: 7, 9) (figure 10.9). Southern blotting experiments using labelled probing oligonucleotides for ET_A-R showed that all ten bands were bound to radiolabelled probes and represent ET_A-R mRNA while with radiolabelled probes for ET_B-R 6 bands appeared suggesting that although only 2 samples produced clearly visible bands in the PCR photograph, 4 more (samples 2, 4, 6 and 8) appear to express ET_B-R mRNA (figure 10.10).

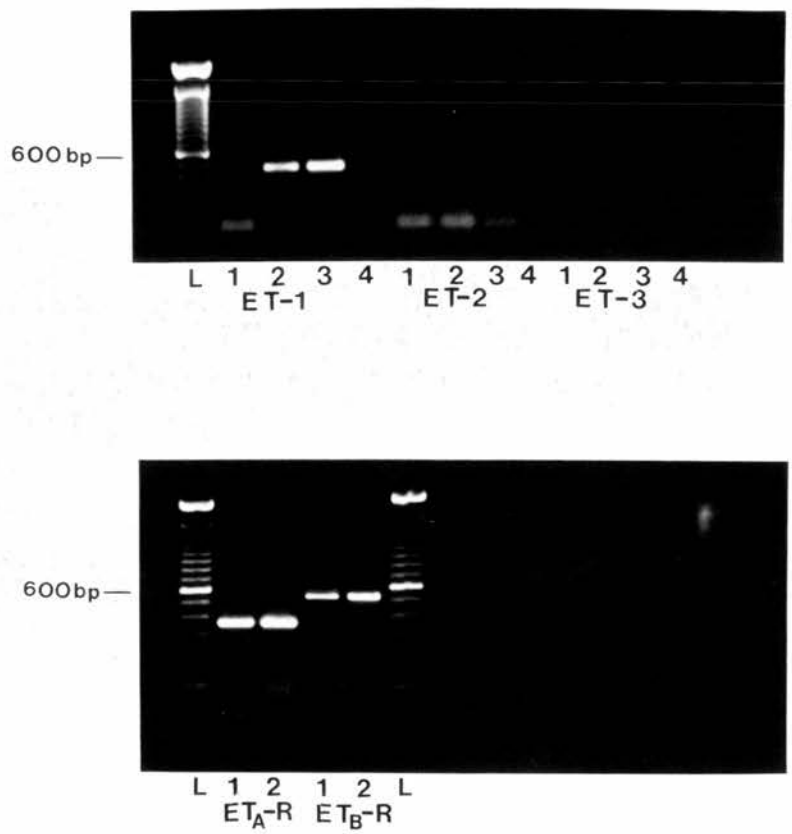


Figure 10.6: Photograph of an agarose gel of 30 cycle PCR products of BRF1 and BRF2 breast fibroblasts. (top) Lane L: 100bp DNA marker; lane 1: negative control BRF1 (no RT step); lane 2: BRF1; lane 3: BRF2; lane 4: negative control, BRF2 (no RT step). (bottom) lane 1: BRF1 cells; lane 2: BRF1cells. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp, ET_A-R: 368bp, ET_B-R: 530bp, ß-actin: 262bp.

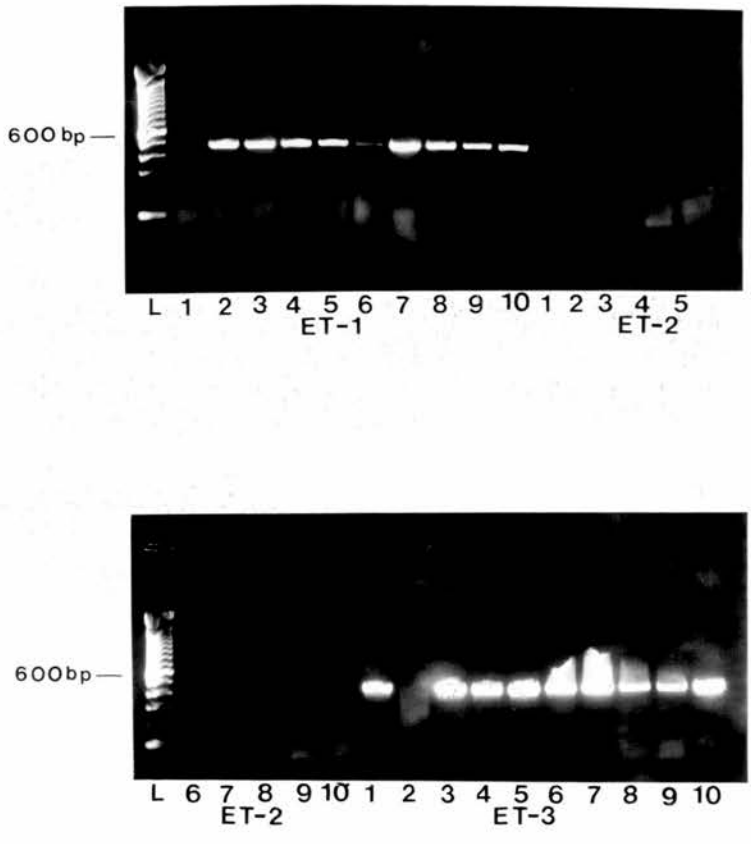
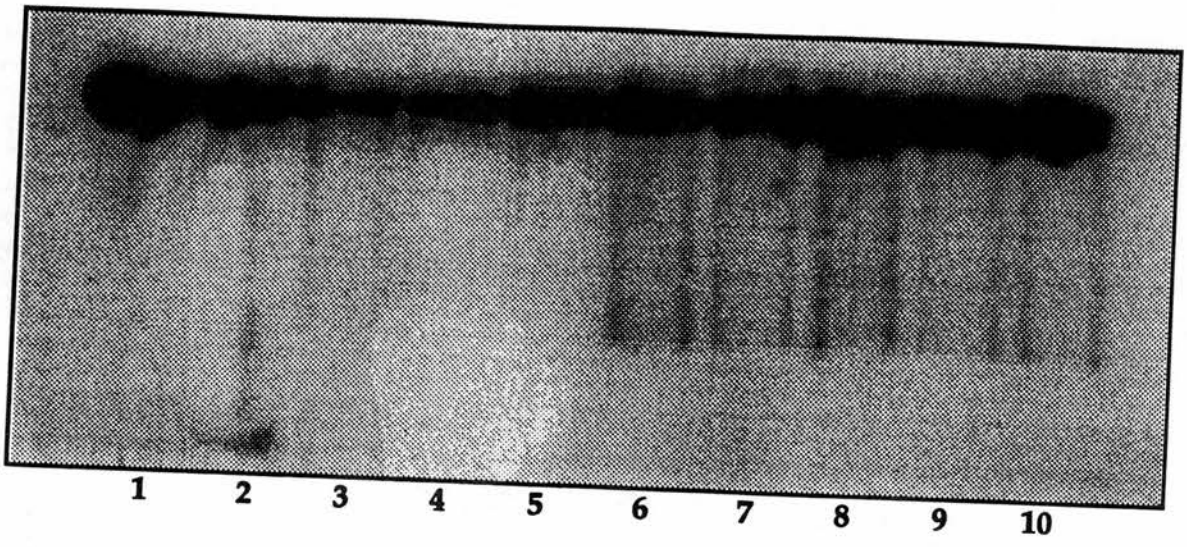
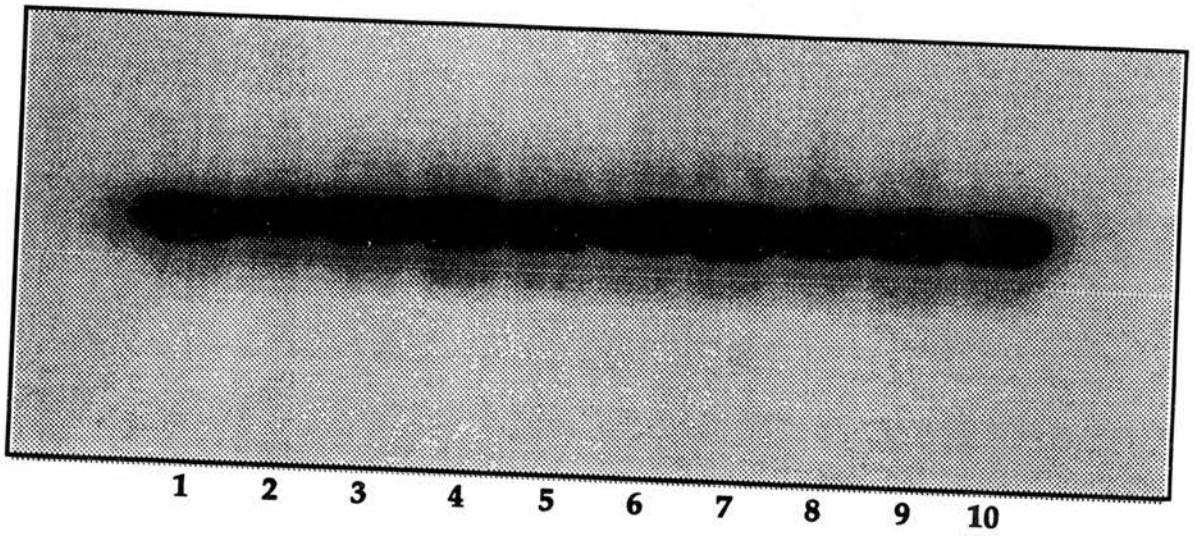


Figure 10.7: Photograph of an agarose gel of 30 cycle PCR products of 10 primary ovarian tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 1-10. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp.



ET-1



ET-3

Figure 10.8: Southern blotting photograph showing the hybridisation of mRNA bands from 10 primary ovarian cancer samples detected by RT-PCR with specific oligonucleotides against ET-1, ET-2 and ET-3.

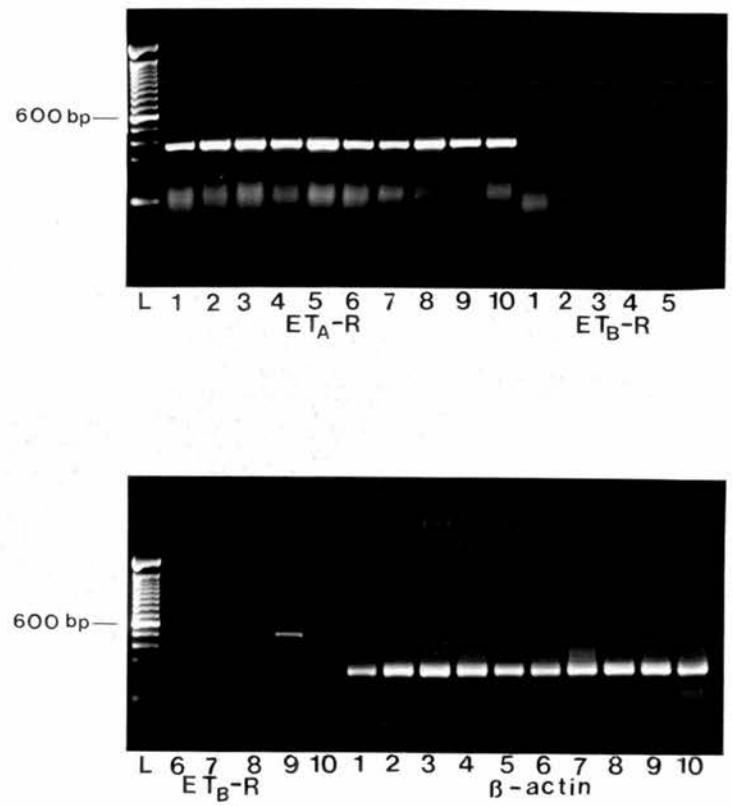
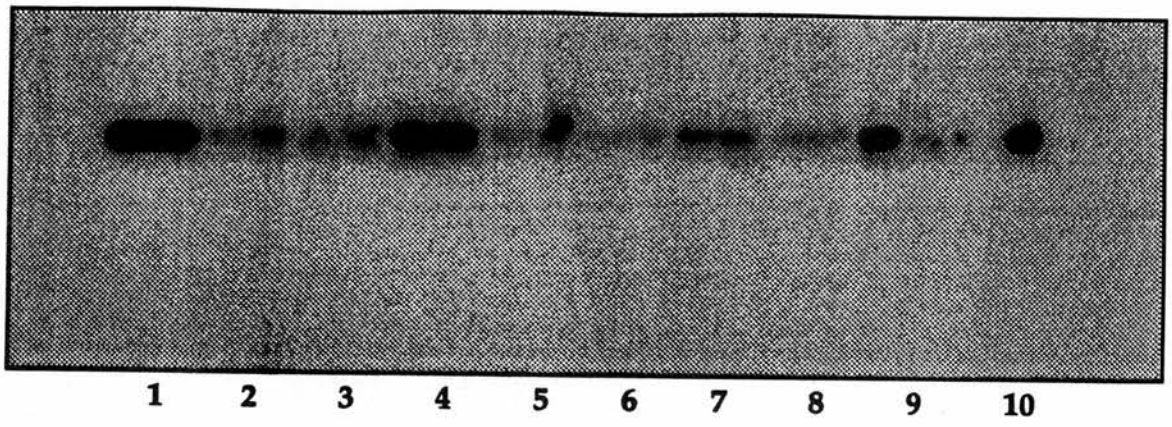
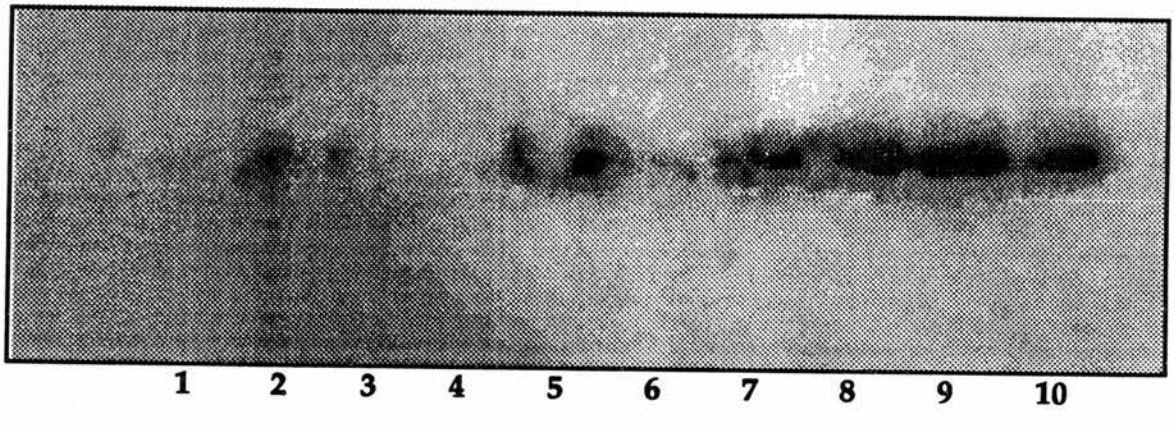


Figure 10.9: Photograph of an agarose gel of 30 cycle PCR products of 10 primary ovarian tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 1-10. The predicted sizes were: ET_A-R: 368 bp, ET_B-R: 530 bp, ß-actin: 262bp.



ET_A-R



ET_B-R

Figure 10.10: Southern blotting photograph showing the hybridisation of mRNA bands from 10 primary ovarian cancer samples detected by RT-PCR with specific oligonucleotides designed against ET_A-R and ET_B-R.

A positive control band at 262bp for ß-actin was observed in all 10 samples. Figure 10.11 shows the expression of ET_A-R mRNA in the identical RNA samples from the same 10 primary tumours, that had (top) and had not (bottom) been through the RT step.

10.2.2 Breast primary tumours

Samples which were obtained from 20 primary breast tumours, were used to prepare RNA and to detect the expression of ET-1, -2 and -3 peptides and ET_A-R, ET_B-R receptors. Figures 10.12 and 10.13 illustrate the expression of mRNA for ET-1 from all 20 tumours while 12 out of 20 expressed mRNA for ET-2 (lanes 1, 2, 3, 5, 6 and 7 in figure 10.12 and lanes 1, 2, 3, 8, 9 and 10 in figure 10.13) and 16 out of 20 mRNA for ET-3 (lanes 2, 4, 5, 6, 7, 9 in figure 10.12 and all 10 in figure 10.13). Southern blotting experiments were performed using radiolabelled probes for ET-1, ET-2 and ET-3 for the same 10 tumour samples shown in figure 10.12 (figure 10.14). These have shown ten bands in identical positions with ET-1, 7 bands for ET-2 (six of which were at identical positions with the ET-2 bands in the PCR gel and a band for sample 8) and 6 bands for ET-3 at the same positions as seen in figure 10.12. Figures 10.15 and 10.16 illustrate the results of the expression of ET_A and ET_B receptors in the same 20 tumour samples. Results demonstrate the expression of ET_A-R and ET_B-R from all 20 primary tumours. Figure 10.17 shows the results of probe labelling using radiolabelled ET_A-R and ET_B-R oligonucleotides which showed that all the bands shown in figure 10.15 represent ET_A-R and ET_B-R mRNA. In figure 10.16 (ET_B-Ra) samples from the same primary tumours were tested as negative controls for the expression of ET_B-R without passing through the RT-step (ET_B-Ra) and no bands were detected suggesting that RNA samples were not contaminated by DNA.

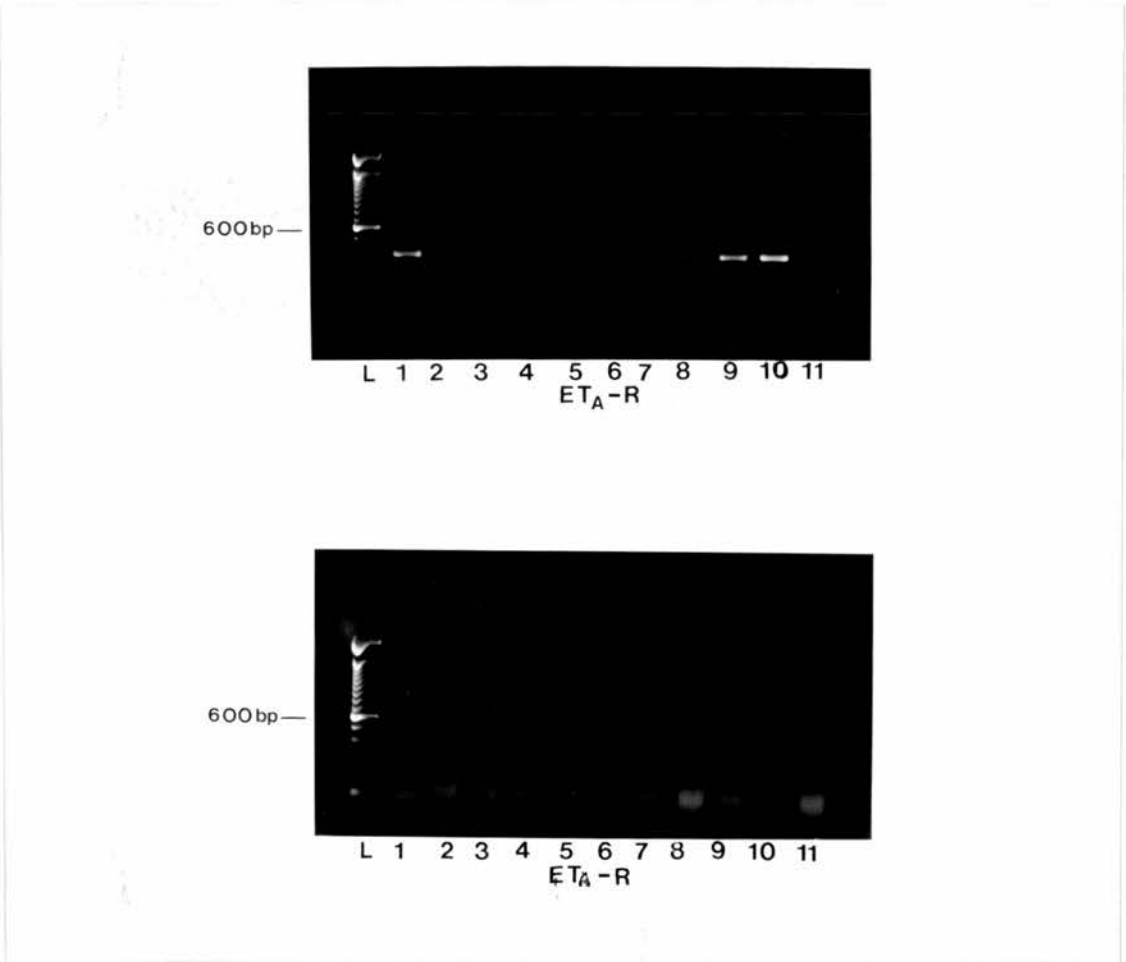


Figure 10.11: Photograph of an agarose gel of 30 cycle PCR products of 10 primary ovarian tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 1-10. (bottom) same as top but samples have not been through the RT step (negative controls). The predicted sizes were: ET_A-R: 368 bp.

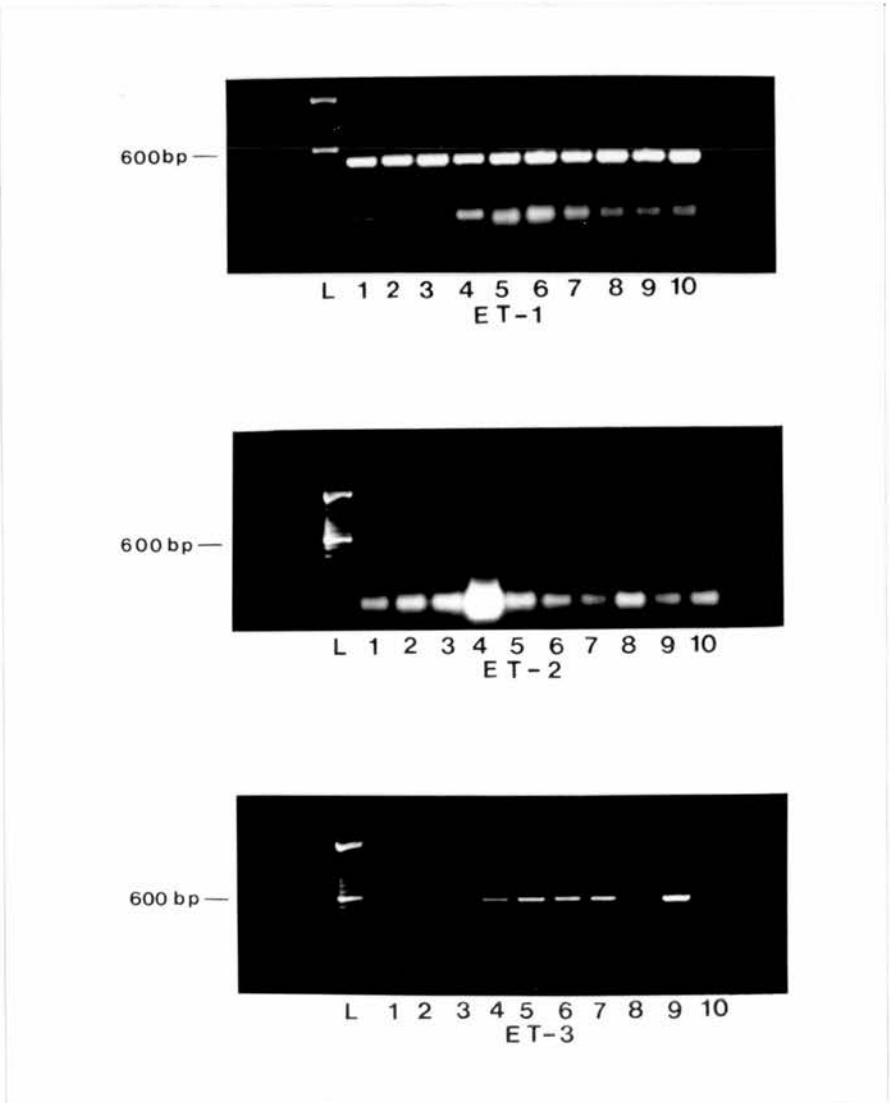


Figure 10.12: Photograph of an agarose gel of 30 cycle PCR products of 10 primary breast tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 1-10. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp.

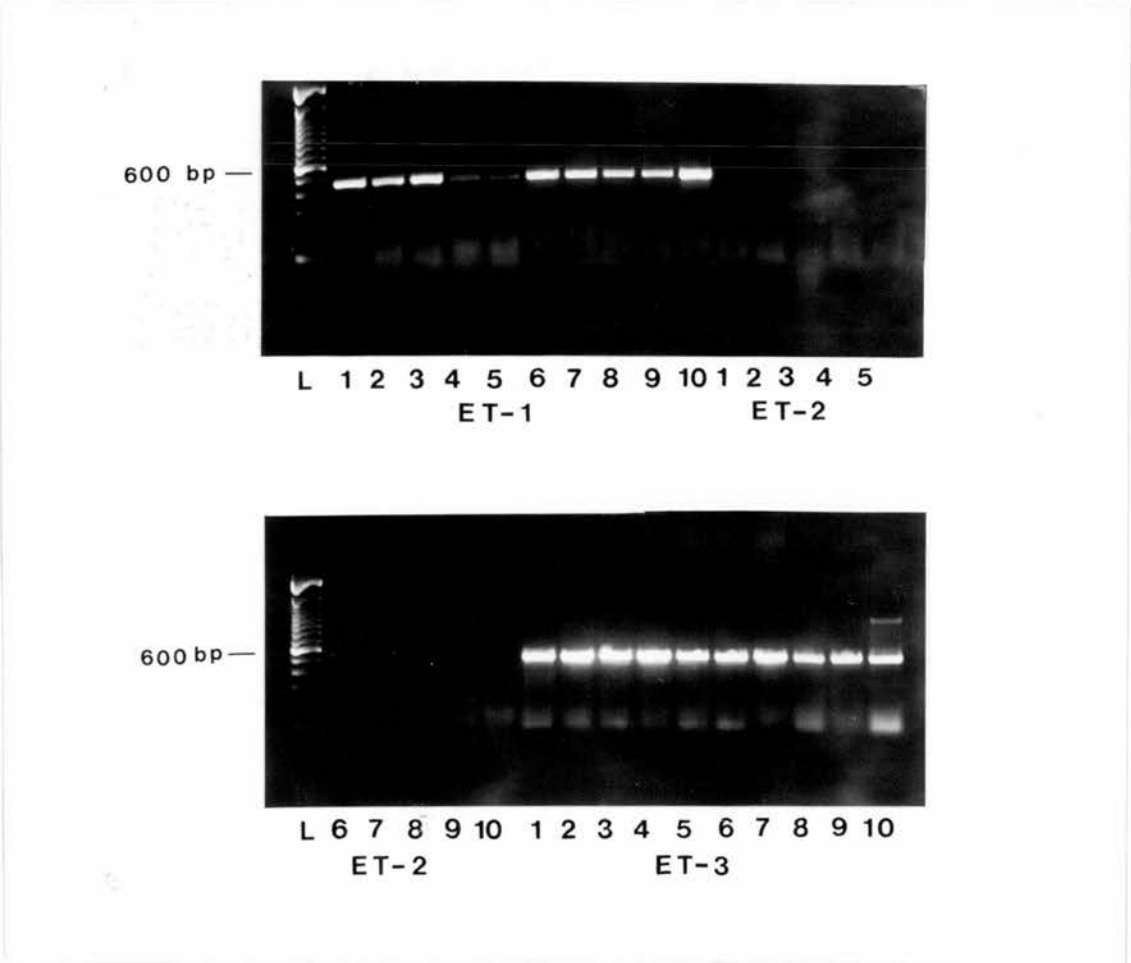
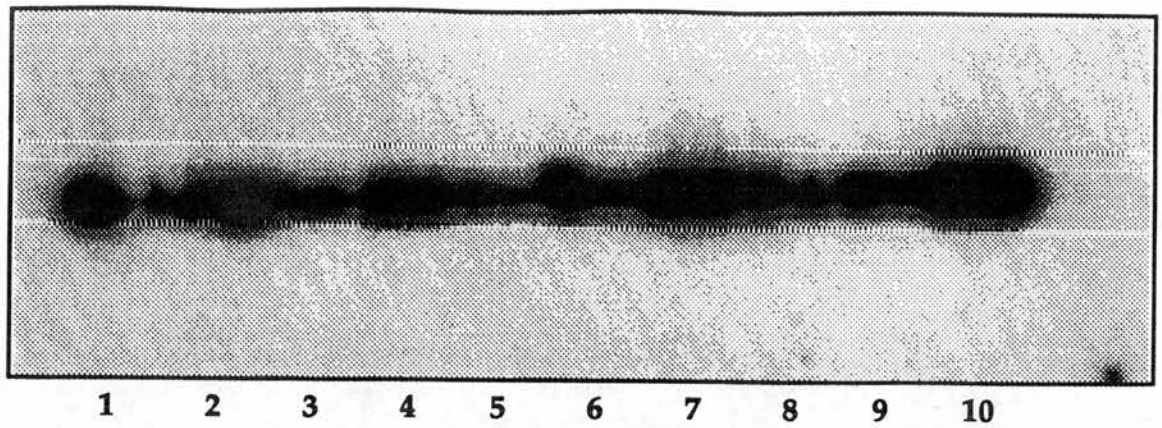
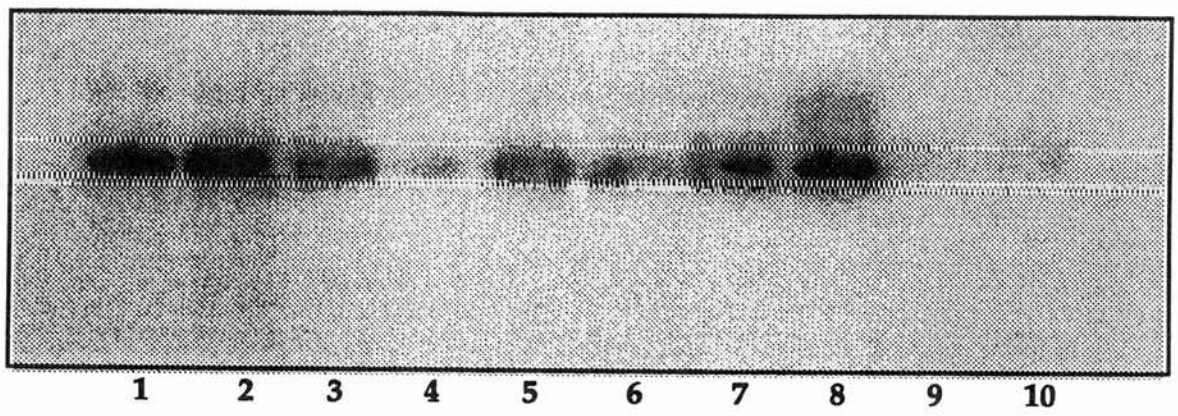


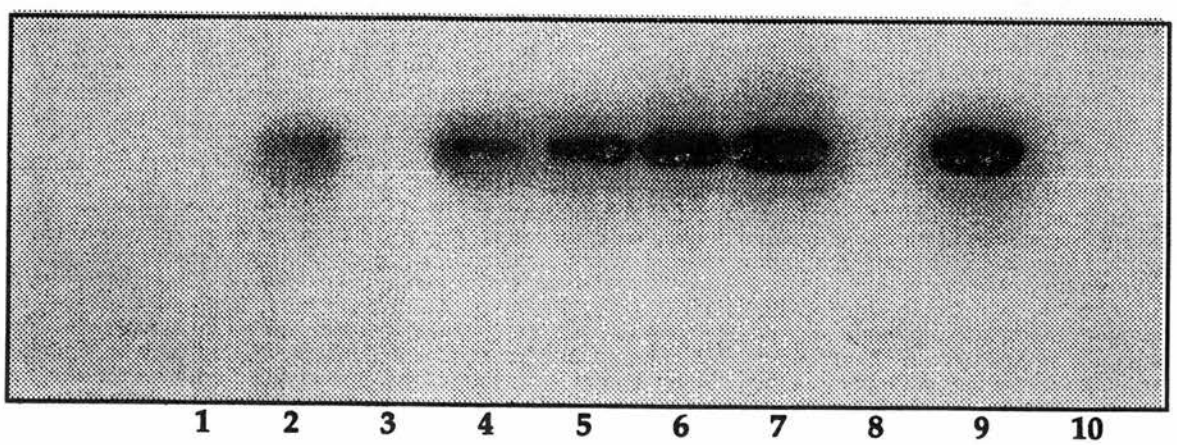
Figure 10.13: Photograph of an agarose gel of 30 cycle PCR products of 10 primary breast tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 11-20. The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp.



ET-1



ET-2



ET-3

Figure 10.14: Southern blotting photograph showing the hybridisation of mRNA bands from 10 primary breast cancer samples detected by RT-PCR with specific oligonucleotides designed against ET-1, ET-2 and ET-3.

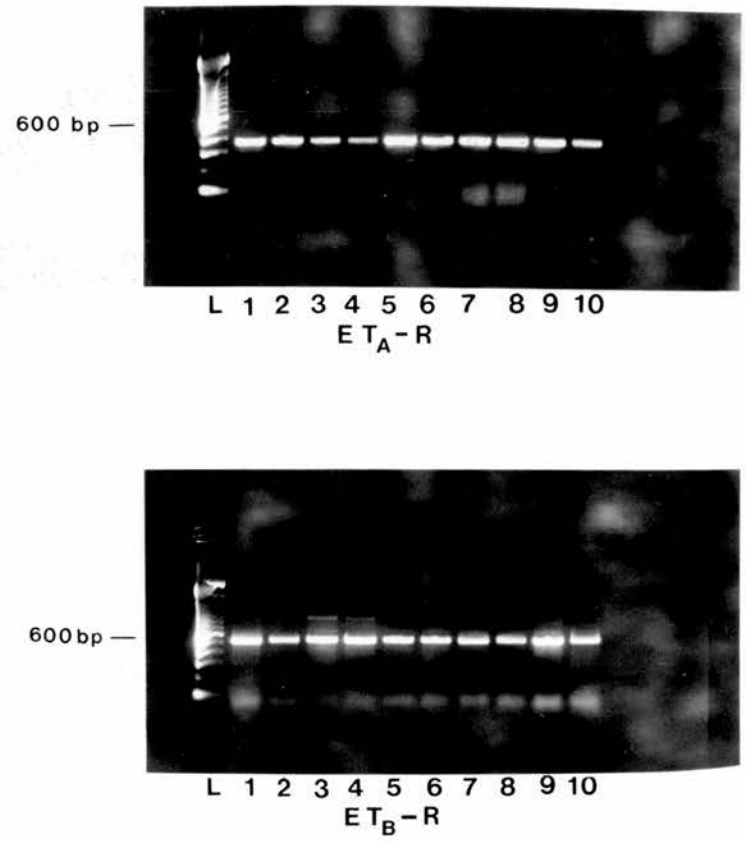


Figure 10.15: Photograph of an agarose gel of 30 cycle PCR products of 10 primary breast tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 1-10. The predicted sizes were: ET_A-R: 368 bp, ET_B-R: 530 bp, ß-actin: 262bp.

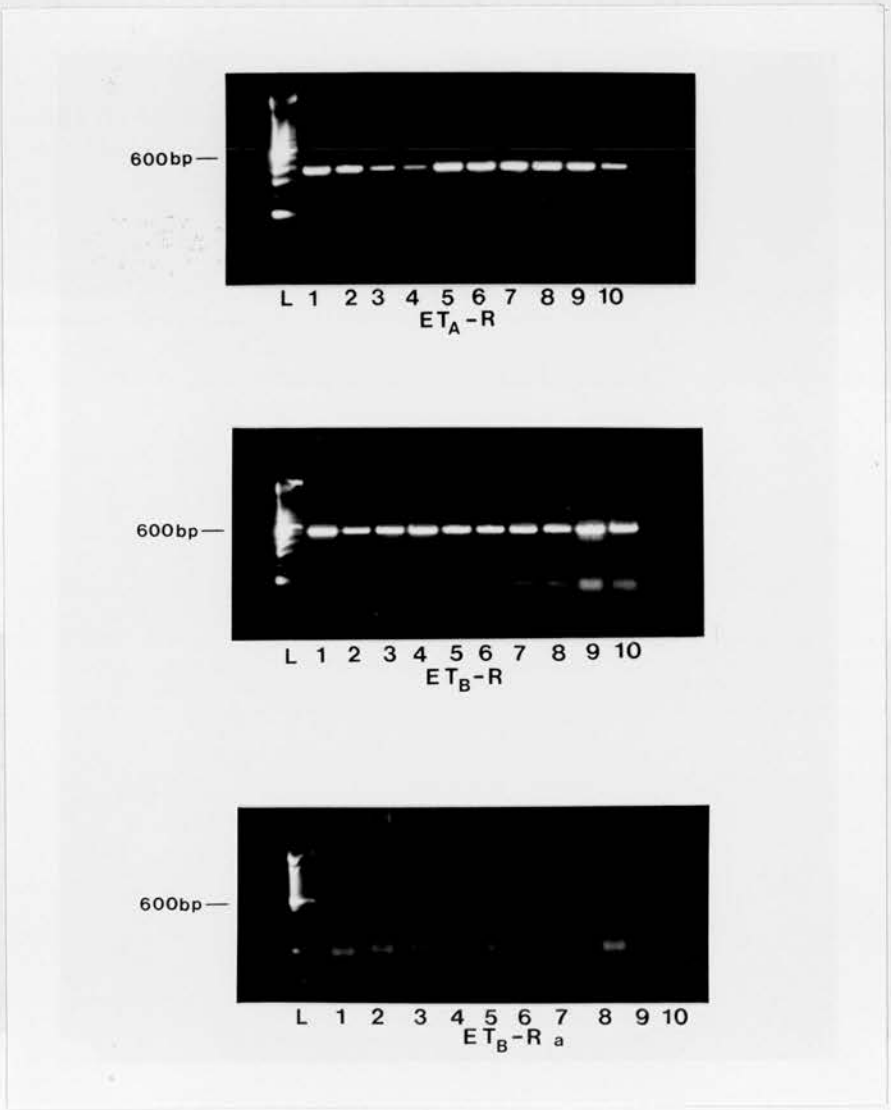
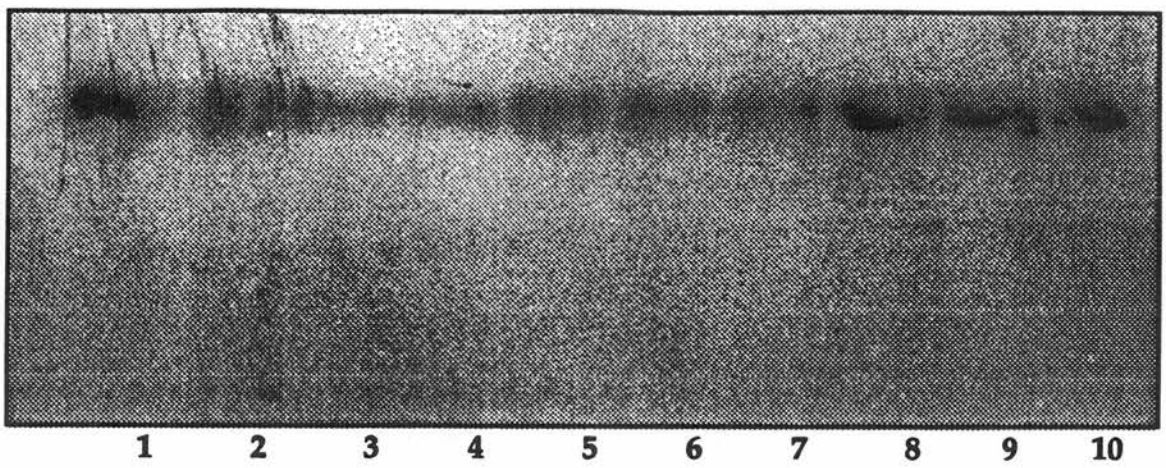
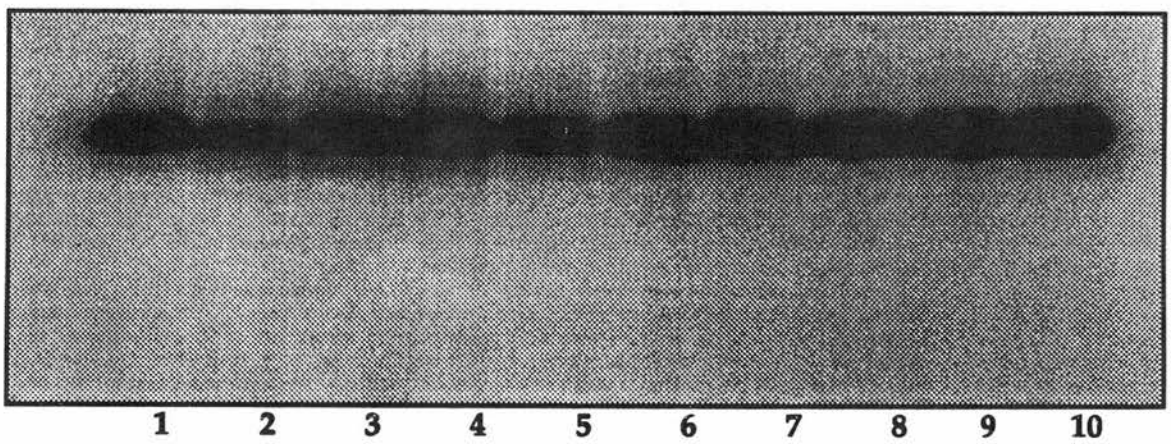


Figure 10.16: Photograph of an agarose gel of 30 cycle PCR products of 10 primary breast tumour samples. (top) Lane L: 100bp DNA marker; lanes 1-10 tumour samples 11-20 (ET_B-R_a: same samples but not taken through the RT step). The predicted sizes were: ET_A-R: 368 bp, ET_B-R: 530 bp, ß-actin: 262bp.



ET_A-R



ET_B-R

Figure 10.17: Southern blotting photograph showing the hybridisation of mRNA bands from 10 primary breast cancer samples detected by RT-PCR with specific oligonucleotides designed against ET_A-R and ET_B-R.

10.2.3 Expression of ET peptides and receptors in normal breast samples

Three histologically proven normal breast samples were used to test ET peptide and receptor expression and to compare results with expression in breast cancer samples. Figure 10.18 shows the expression of ET-1 in all 3 samples, that of ET-2 and ET-3 in 2 samples (lanes: 1, 2 for each). In the same figure, mRNAs for both ET-receptors and ß-actin were shown to be expressed in all 3 samples. Negative controls (lane 4; sample 1 for ET-1 and ET-2, sample 2 for ET-3 and ET_A-R and sample 3 for ET_B-R and ß-actin) did not show any bands.

Table 10.1 summarises the results of the expression of ETs and ET-receptors presented in this chapter.

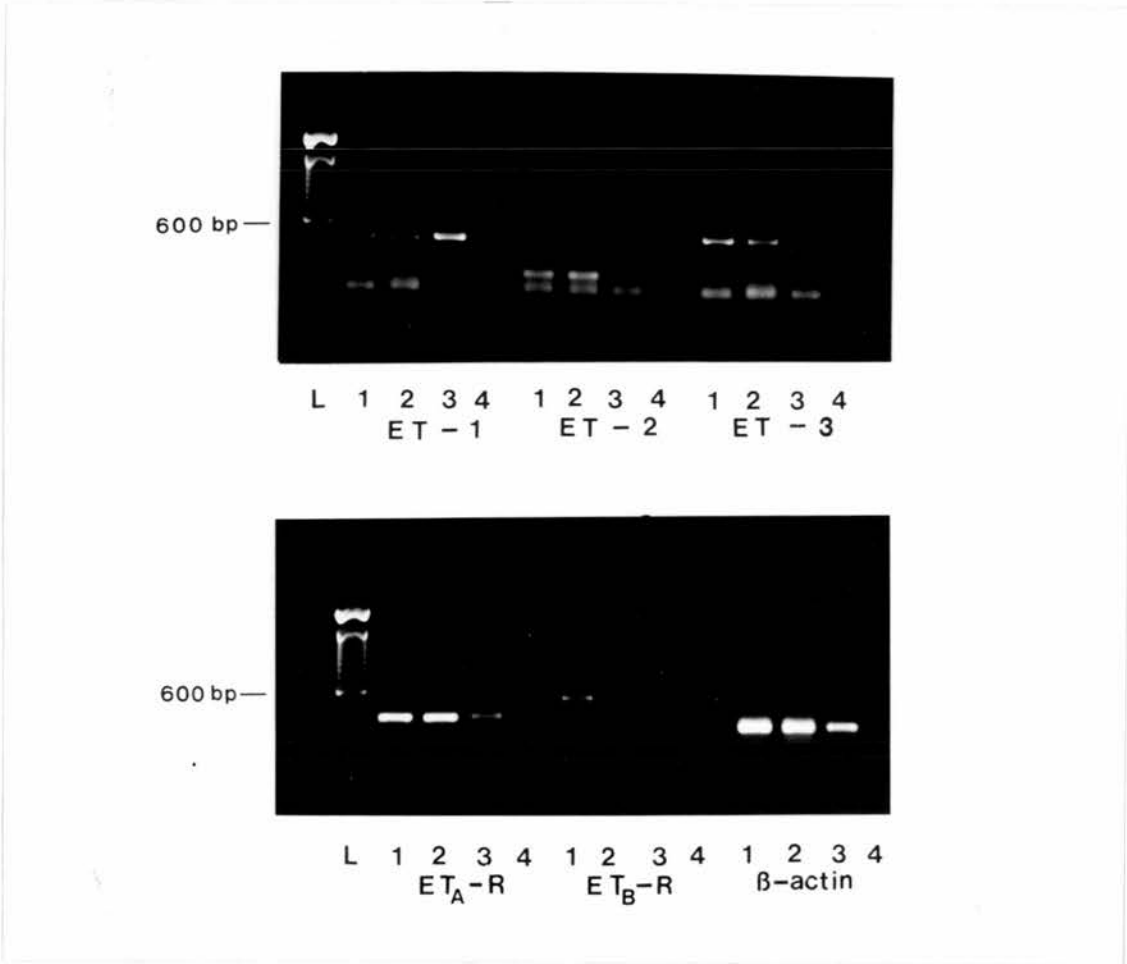


Figure 10.18: Photograph of an agarose gel of 30 cycle PCR products of 3 histologically proven normal breast samples. Lane L: 100bp DNA marker; lanes 1-3 samples 1-3; lane 4: negative control (not through the RT step). The predicted sizes were: ET-1: 582 bp, ET-2: 197 bp, ET-3: 493bp, ET_A-R: 368 bp, ET_B-R: 530 bp, ß-actin: 262bp.

mRNA source	ET-1	ET-2	ET-3	ET_A-R	ET_B-R
ovarian cancer cells	2/2	0/2	2/2	2/2	2/2
ovarian fibroblasts	3/4	0/4	0/4	3/4	4/4
breast cancer cells	4/4	0/4	3/4	0/4	3/4
breast fibroblasts	2/2	0/2	0/2	2/2	2/2
ovarian tumours	10/10	6/10	9/10	10/10	2/10
breast tumours	20/20	12/20	16/20	20/20	20/20
normal breast	3/3	2/3	2/3	3/3	3/3

Table 10.1: Summary of the results of the expression of ET-1, ET-2, ET-3, ET_A-R and ET_B-R from ovarian and breast cancer cell lines, fibroblasts, primary tumour samples and normal breast samples.

10.3 Discussion

Results presented in this chapter show the mRNA expression for ET-1, ET-2 and ET-3 peptides and the two ET-receptors (ET_A-R and ET_B-R) in ovarian and breast cancer cells and fibroblasts as well as from 10 ovarian, 20 breast primary tumour samples and 3 normal breast samples.

RT-PCR technology using RNA from PEO4 and PEO14 ovarian cancer cell lines demonstrated the expression of mRNA for ET-1 and ET-3 in these cell lines. These results are consistent with observations in chapter 4 where it was concluded that the same cell lines secrete immunoreactive ET-1-like material. Because mRNA for ET-3 was detected by RT-PCR and the cross-reactivity of ET-3 with the radioimmunoassay used for ET-1 detection, was calculated to be 8.3% it is possible that ET-3 could account for part of the ET-1-like material detected by RIA assuming that the detected mRNA was translated into protein. RT-PCR also detected the expression of mRNA for both ET receptor subtypes in these two cell lines. Such results would be inconsistent with the conclusions stated in previous chapters which suggested that PEO14 cells, express the ET_A-R type. However, for PEO4 cells results shown in previous chapters suggested the expression of ET_A-R and the possible expression of a small amount of ET_B-R which would be consistent with the expression of mRNA for both receptors.

Results from the RT-PCR experiments using RNA from 4 ovarian fibroblast cell lines suggested the expression of mRNA for ET-1 and ET-3. Such results are in contrast with data from the radioimmunoassay study (chapter 4) in which conditioned media from 3 of these cell lines (PEO12F, PEO14F and PEO27F) did not appear to contain ET-1-like material. However, the detection of ET-1-like peptide would only be possible if the mRNA was translated into protein and it may be that levels of ET were below the level of sensitivity in the assay.

RT-PCR analysis also demonstrated the expression of mRNA for both types of receptors in PEO12F, PEO14F and PEO27F fibroblast cell lines (except for ET_A-R in PEO9F). These results would be in accordance with

the expression of ET_A and ET_B receptors in all these cell lines reported in previous chapters.

Results from the breast cancer cell lines suggested the expression of mRNA for ET-1 and ET-3 in all 4 cancer cell lines (except for ET-3 mRNA in T47D cells). Results from the radioimmunoassay experiments using conditioned media from MDA-MB-231, T47D and ZR-75-1 but not MCF-7 cells suggested the expression of ET-1-like material by all 3 cancer cell lines which is consistent with what is observed at the RNA level. The cross-reactivity of ET-3 with the RIA assay could mean that a component of the ET-1-like material is ET-3. Results from RT-PCR also suggested that none of those 4 breast cancer cell lines expressed mRNA for ET-receptors which is consistent with the results presented in previous chapters.

Breast fibroblast cell lines BRF1 and BRF2 were shown to express mRNA for ET-1 but not ET-2 or ET-3. The expression of ET-1 mRNA is in contrast with the absence of ET-1-like material from conditioned media of both cell lines (chapter 4) which might suggest that the RNA detected is not translated into protein. However, the expression of mRNA for both ET_A-R and ET_B-R were in accordance with results from the binding experiments and growth results analysed in previous chapters.

RT-PCR analysis suggested the expression of ET-1 (in 10/10), ET-2 (in 6/10), ET-3 (in 9/10), ET_A-R (in 10/10) and ET_B-R (in 10/10) in primary ovarian tumour samples. Since the tumours consist of mainly epithelial cells then these results are in accordance with those observed in the ovarian cancer cell lines.

Results from primary breast tumour samples showed the expression of mRNA for all 3 ET-peptides (in 20/20, 12/20 and 16/20 for ET-1, ET-2 and ET-3 respectively) and both ET-receptors (in all 20 samples). Although the mRNA expression for ET peptides was seen in breast cancer cell lines no detection of mRNA for receptors was detected. This could be explained by the possible presence of fibroblasts within the tumour samples.

The use of normal breast samples for the detection of ET-peptide and

receptor suggested that the presence of mRNA for ET-1, -2 and -3 and both ET-receptors. Therefore no difference in terms of expression was observed between normal and cancer samples. However, results are consistent with the suggestion that differences are quantitative and breast cancer cell lines secrete higher amounts of ET-1 than either benign or normal breast cells (Yamashita et al., 1991).

Chapter 11: Expression of ETs in ovarian and breast primary tumour samples as detected by immunohistochemistry

The expression of ETs in ovarian and breast cancer tumours was investigated using paraffin-fixed sections from 15 ovarian and 15 breast primary tumour samples. Details regarding the methodology and the antibodies used are described in section 2.2.8.

11.1 ET expression in ovarian tumours

i) ET-1

The results on the expression of ET-1, ET-2 and ET-3 in ovarian primary tumour samples are summarised in table 11.1.

ET-1 expression was investigated in 15 primary tumour samples and in both tumour and stromal areas. Four tumour samples exhibited negative staining both in tumour and stromal compartments. From the 11 tumour samples that stained positively for ET-1 expression in the tumour areas, two were strongly positive, five were weakly positive and 4 samples showed borderline staining. Stromal ET-1 staining was observed in 6 of the 15 samples and its intensity was lower or similar to levels observed in the tumour areas. Staining was generally diffuse, mostly cytoplasmic and occasionally granular. A typical section stained positively for ET-1 is shown in figure 11.1.

ii) ET-2 and ET-3

The expression patterns of ET-2 and ET-3 were investigated in 7 primary ovarian tumour samples. Positive staining was diffuse and mainly cytoplasmic. ET-2 expression was observed in the tumour compartment in 5 of the 7 samples, and staining was weakly positive in 3 and borderline in the other 2 samples. Only one of the samples showed borderline staining in the stromal areas. ET-3 expression was positive in the tumour areas of 5 primary tumour samples, 2 of which exhibited weak staining and 3 borderline staining. ET-3 staining in the stromal areas of the tumour samples was observed at borderline levels in 2 samples.

Sample	ET-1		ET-2		ET-3		negative control	
	tumour	stroma	tumour	stroma	tumour	stroma	tumour	stroma
HOV 1	+	±	+	-	+	-	-	-
HOV2	+	-	ND	ND	ND	ND	-	-
HOV3	+	-	ND	ND	ND	ND	-	-
HOV4	++	-	-	-	±	-	-	-
HOV5	+	-	+	-	±	-	-	-
HOV6	±	+	-	-	-	-	-	-
HOV7	±	±	±	±	+	±	-	-
HOV8	++	++	+	-	-	-	-	-
HOV9	-	-	ND	ND	ND	ND	-	-
HOV10	±	±	ND	ND	ND	ND	-	-
HOV11	+	±	±	-	±	±	-	-
HOV12	±	-	ND	ND	ND	ND	-	-
HOV13	-	-	ND	ND	ND	ND	-	-
HOV14	-	-	ND	ND	ND	ND	-	-
HOV15	-	-	ND	ND	ND	ND	-	-

Table 11.1: Results of the expression of ET-1, -2 and -3 from ovarian primary tumour samples (HOV). Intensity of staining is expressed as: strong positive (++), weak positive (+), borderline (±) and negative(-) (ND: not done).

Figure 11.1: Positive staining of an ovarian primary tumour section with ET-1 antibody.

Examples of positive staining for ET-2 and ET-3 are shown in figures 11.2 and 11.3.

Sections which were treated with tris-buffer instead of ET antibodies (negative controls) were stained negatively as shown in figure 11.4.

11.2 ET expression in breast tumours

Table 11.2 summarises the results of the expression of ET-1, ET-2 and ET-3 in breast primary tumours.

i) ET-1

The expression of ET-1 in the tumour and stromal areas of 15 primary breast tumour samples was investigated.

Two tumour samples stained negatively in both tumour and stromal areas. ET-1 was detected in the tumour areas of 13 of the 15 tumours, 5 of which stained strongly positive, 5 others weakly positive while 3 samples exhibited borderline staining. Weak ET-1 staining in the stromal areas was observed in one sample, borderline staining in 4 samples and no positive staining in the rest of the samples. An example of positive ET-1 staining is shown in figure 11.5.

ii) ET-2 and ET-3

The expression of ET-2 and ET-3 was investigated in 6 primary breast tumour samples. Two breast tumour samples exhibited weak staining for both ET-2 and ET-3 in the tumour but not in the stromal areas and a third sample showed borderline staining for ET-3 in the tumour area. Examples for ET-2 and ET-3 positive staining are shown in figures 11.6 and 11.7 respectively.

Control samples (treated with tris-buffer instead of ETs) exhibited no positive staining.

Figure 11.2: Positive staining of an ovarian primary tumour section with ET-2 antibody.

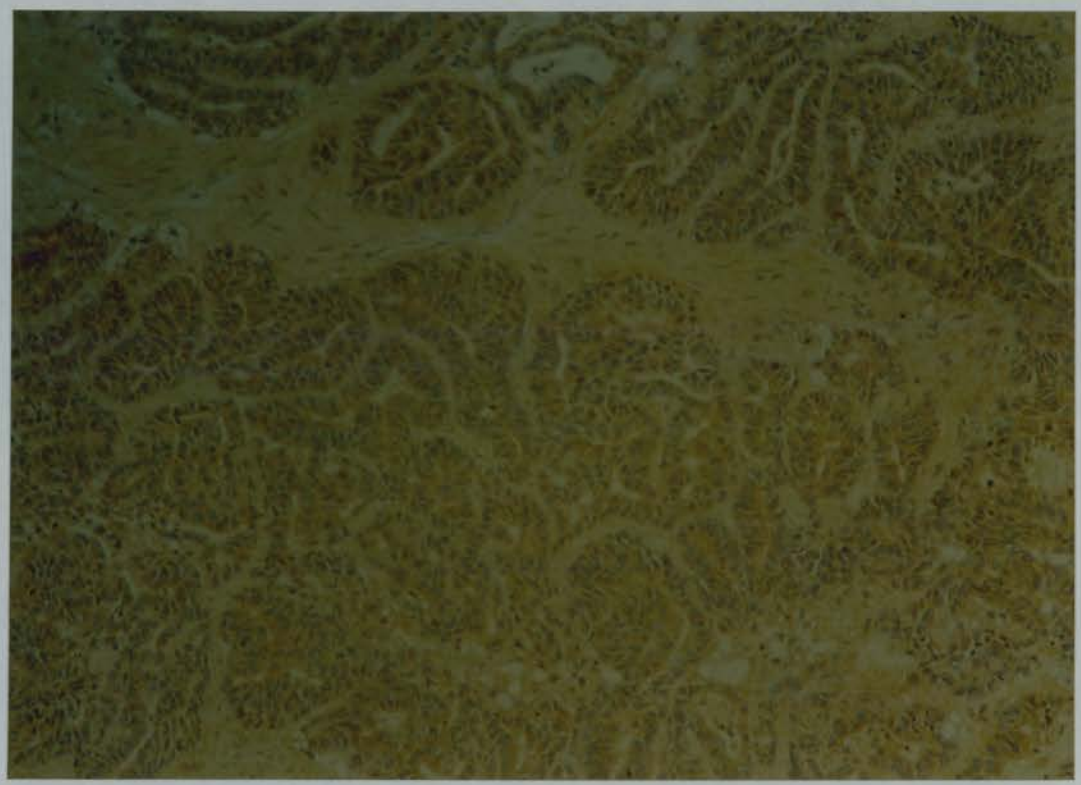


Figure 11.3: Positive staining of an ovarian primary tumour section with ET-3 antibody.

Figure 11.4: Absence of staining of an ovarian primary tumour (tris buffer used as negative control).

Sample	ET-1		ET-2		ET-3		negative control	
	tumour	stroma	tumour	stroma	tumour	stroma	tumour	stroma
BC1	+	-	+	-	+	-	-	-
BC2	++	+	ND	ND	ND	ND	-	-
BC3	±	-	-	-	-	-	-	-
BC4	±	±	ND	ND	ND	ND	-	-
BC5	-	-	-	-	-	-	-	-
BC6	++	±	ND	ND	ND	ND	-	-
BC7	++	±	ND	ND	ND	ND	-	-
BC8	++	-	ND	ND	ND	ND	-	-
BC9	+	-	ND	ND	ND	ND	-	-
BC10	+	-	+	-	+	-	-	-
BC11	++	±	ND	ND	ND	ND	-	-
BC12	±	-	-	-	-	-	-	-
BC13	-	-	ND	ND	ND	ND	-	-
BC14	+	-	-	-	±	-	-	-
BC15	+	-	ND	ND	ND	ND	-	-

Table 11.2: Results of the expression of ET-1, -2 and -3 from breast primary tumour samples (BC). Intensity of staining is expressed as: strong positive (++), weak positive (+), borderline (±) and negative(-) (ND: not done).

Figure 11.5: Positive staining of an breast primary tumour section with ET-1 antibody.

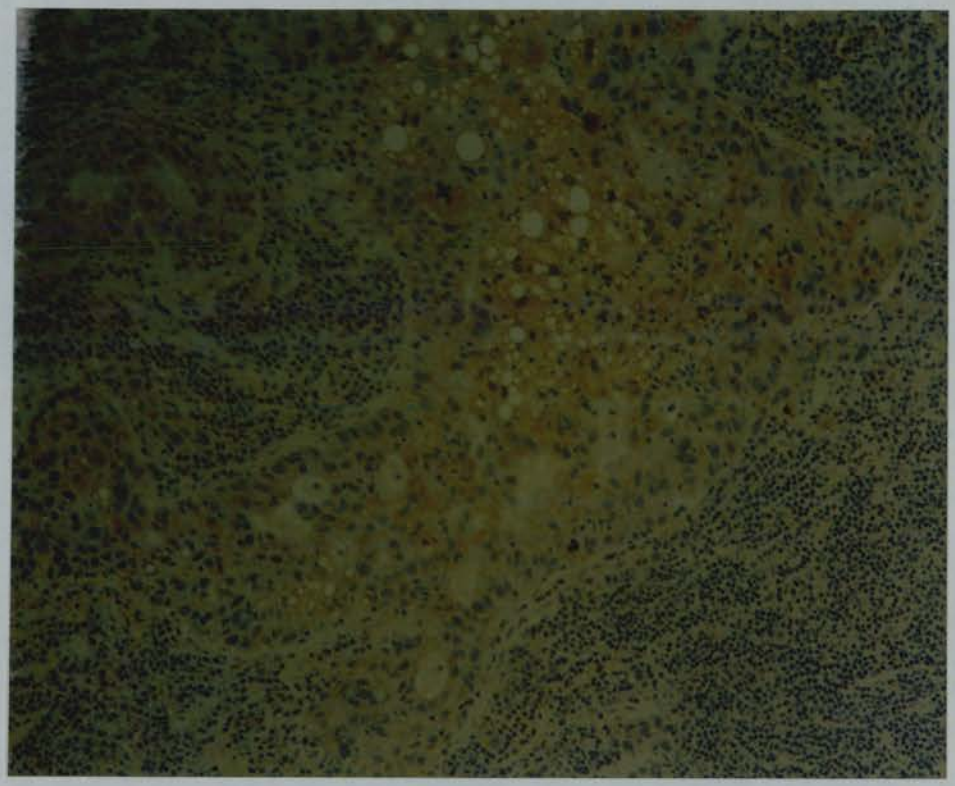


Figure 11.6: Positive staining of an breast primary tumour section with ET-2 antibody.

Figure 11.7: Positive staining of an breast primary tumour section with ET-3 antibody.

11.3 Discussion

The expression of ETs in ovarian and breast primary tumour samples was investigated immunohistochemically using commercially available ET-1, ET-2 and ET-3 antibodies who according to our radioimmunoassay studies show small crossreactivities with the other ET peptides.

In ovarian tumours, ET-1 was detected in the tumour areas of the majority of samples (11/15) while some stromal staining was observed in 6 of the same 11 samples. ET-2 and ET-3 weak and borderline staining appeared in 5/7 tumour samples and only in the tumour areas (with the exception of borderline staining in 1 sample for both ET-2 and ET-3).

Radioimmunoassay data (chapter 4) for ovarian cancer and fibroblast cell lines suggested the expression of ET-1-like material from cancer cells but not fibroblasts. Results presented in this chapter seem to be in accordance, showing that most of the staining was observed in the epithelial areas of the samples with generally more diffuse staining in the stromal areas. Thus the weak staining observed in the stromal areas of these samples could represent ET peptides which originated in cancer cells and have been transported to stromal areas. As described in previous chapters, receptors for ETs are expressed in both ovarian epithelial and fibroblast cells and both cell types in vivo may therefore be under ET regulation.

In breast tumours, ET-1 expression was observed in the epithelial components of the majority of samples (13/15) while predominantly borderline staining was observed in the stromal compartments of 5/15 samples. ET-2 and ET-3 expression was observed in 2 and 3 (respectively) of the 6 samples tested. These results suggest that ETs are mainly expressed in breast tumour compared to stromal cells and these observations are in accordance with previously published data (Baley et al., 1990). RIA results presented earlier in this thesis, also indicated the production of significant amounts of ET-1-like material from epithelial breast cancer cells but not fibroblasts (chapter 4). Baley et al., (1990) have also suggested that ET receptors are expressed in breast fibroblasts but not in epithelial tumour cells. Binding experiments shown in chapter 5

their biological effects, the peptides need to interact with the fibroblasts. Such a phenomenon could explain (as in ovarian primary tumour samples), the generally weaker staining in the stromal areas of some breast tumour samples.

Five of the HOV samples that were used in this immunohistochemistry study (HOV 1, 4, 5, 7 and 11) came from the same tumour as samples used for the RT-PCR experiments. Immunohistochemistry data showed that 5/5 samples stained positively for ET-1, 4/5 for ET-2 and 5/5 for ET-3. PCR data showed that the same 5 samples all expressed ET-1 ET-2 and ET-3 mRNA.

Results from five of the BC samples used for immunohistochemistry (BC1, 3, 5, 10 and 14) regarding the expression can be compared with results of the PCR experiments since samples from the same primary tumours were used. In this chapter it was shown that 4/5 stained positively for ET-1, 2/5 for ET-2 and 3/5 for ET-3. RT-PCR data showed that 5/5 expressed ET-1 mRNA, 3/5 ET-2 mRNA (the same as in immunohistochemistry plus BC14) and 5/ 5 samples expressed ET-3.

For some of the primary tumour samples, mRNA was detected for a paticular ET peptide but no staining was observed with immunohistochemistry. Since there were no HOV or BC tumour samples which stained positively for any of the ET peptides but showed no expression of mRNA, it is possible that the mRNA observed was not translated into protein. However, such results could also be explained by the very high sensitivity of the PCR method.

These data show that results from the two methods were consistent (accounting for the fact that one shows expression in terms of mRNA and the other in terms of protein).

In general, and despite the restricted number of tumour samples used (which did not allow detailed analysis and correlation of tumour types with expression of the peptides) and the absence of proven commercially available ET-receptor antibodies, results presented above represent the first data suggesting the expression of ET-1, ET-2 and ET-3 in ovarian

first data suggesting the expression of ET-1, ET-2 and ET-3 in ovarian tumours and the restricted expression of ET-2 and ET-3 in breast primary tumours.

However, results obtained by these studies should be handled with caution due to the fact that the specificity of the human monoclonal ET-1, ET-2 and ET-3 antibodies was not tested and that the negative control used in these experiments was tris buffer. Other confirmatory studies should have been undertaken. These include employing negative controls such as cheking the positivity of blood samples from the same species as the antibodies were raised against but from animals that had not been immunised with the antibody and checking the specificity of the antibodies by western blot analysis.

Chapter 12: Conclusions

In the previous chapters of this thesis, data on the expression of the ETs, their receptors and their effects on the growth of ovarian and breast cancer have been presented and discussed. In this final chapter an attempt has been made to combine these results and provide models of these ET in these two diseases.

12.1 Endothelins in ovarian cancer

The basic aim of this study was to determine whether ETs play a role on the growth of ovarian and breast cancer. Growth regulation of cells by a particular peptide requires: a) the presence of sufficient concentrations of the peptide, b) the presence of cellular receptors to mediate the effects of the peptide and c) an effect on growth (generally mitogenesis) following the binding of the peptide to receptors.

In this study it has been shown that the PEO14 and PEO4 ovarian carcinoma cell lines express mRNA for ET-1 and ET-3 and secrete ET-1 like peptide. ET receptors are expressed in both these cell lines at the mRNA and protein levels and these appear to be functional in so far as addition of both ET-1 or ET-2 are mitogenic to these two cell lines. The concentrations of ET-1-like peptide produced by PEO14 cells are 10-fold greater than the concentration produced by PEO4 cells and are predicted to be growth stimulatory in that exogenous addition of picomolar levels stimulated growth of PEO14 cells. It is feasible therefore that an autocrine growth stimulatory loop is present in PEO14 cells but not necessarily in the PEO4 line. Consistent with this hypothesis, interruption of the loop at the receptor level (ET_A-R) either by use of a receptor blocking antagonist (BQ123) or an antisense oligonucleotide (inhibiting the synthesis of the receptor) produced growth inhibition of cells growing in a serum-free environment and in which the only source of endothelin cells themselves. Such effects were not observed in the PEO4 cell line consistent with a lower level of production being insufficient to produce mitogenesis.

Experiments with ovarian fibroblast cell lines indicated that these cells produce ET-1 mRNA but not detectable levels of protein. However these cells do possess ET receptors as detected at both the mRNA and protein levels and were growth stimulated by all 3 ET isoforms. To examine the interaction of these 2 cell types, a co-culture model was used. The results obtained suggested that the endothelins secreted by the epithelial cancer cells produced growth stimulation in the fibroblasts and these effects could be partially blocked by ET receptor antagonists. These data indicate that in addition to autocrine control, paracrine regulation may also apply. In support of the view that these models are relevant to growth regulation in primary tumours, RT-PCR and immunohistochemical studies of a series of primary tumours indicated the expression of all 3 forms of ET in the majority of primary together with both forms of receptors as detected by RT-PCR.

A single report has previously been published on the role of ETs in ovarian cancer (Bagnato et al., 1995). These investigators demonstrated the expression of immunoreactive ET-1 by ovarian carcinoma cell lines, expression of ET-receptors and growth stimulation by ET-1 supporting the findings described in this study.

Combining these results, a potential model of the effect of ETs on the growth of ovarian cancer has been proposed (figure 12.1). According to this, ET peptides secreted by the epithelial cancer cells can act through an autocrine system and stimulate the growth of the cancer cells while the same peptides acting through an alternative or additional paracrine system also stimulate the growth of surrounding fibroblasts. Since it was demonstrated that other molecules (bombesin, IL-6) stimulate the production of ETs from cancer cells (chapter 4) and that ETs in co-culture experiments accounted for a partial stimulation of the growth of cancer cells and fibroblasts, it is almost certain that other factors modulate the endothelin system through different mechanisms of action.

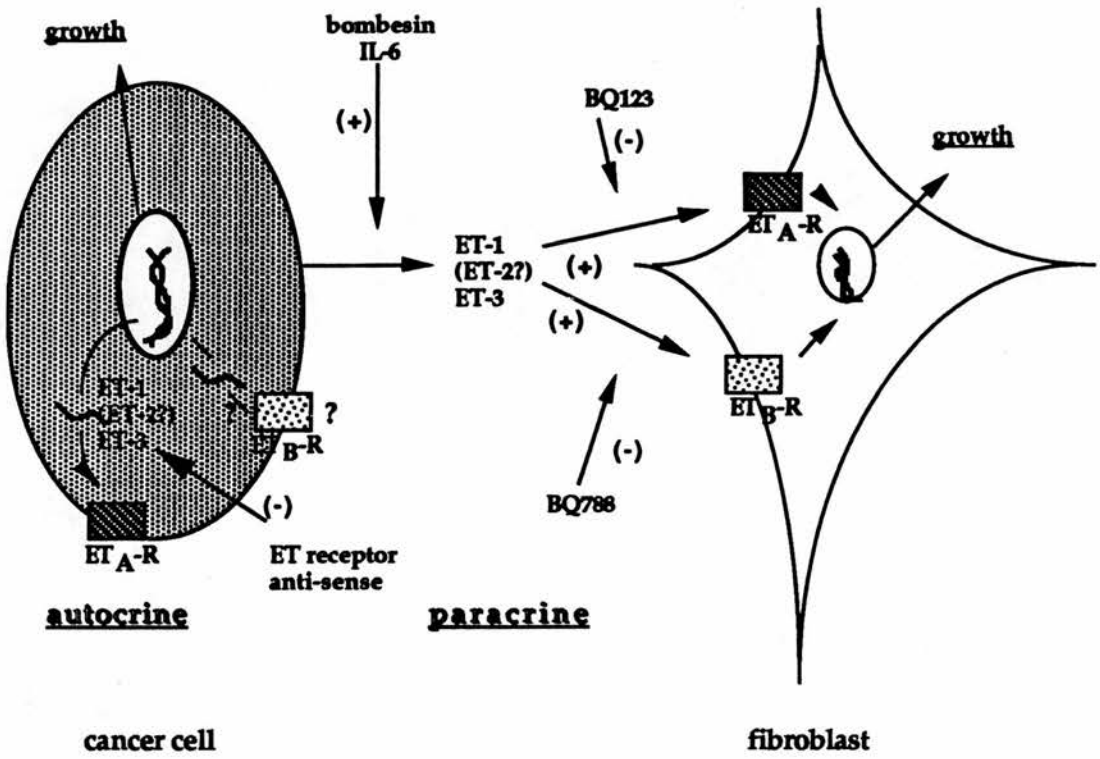


Figure 12.1: A diagrammatic representation of the suggested model of the involvement of ETs on the growth of ovarian cancer cells and fibroblasts. ETs which are shown to be secreted exclusively by the epithelial cells, can bind to both ET_A-R and ET_B-R ET-receptors on fibroblasts and exert mitogenic effects through a paracrine system of action and such effects can be blocked using specific ET-receptor antagonists (BQ123 for ET_A-R and BQ788 for ET_B-R). However, ET peptides released by cancer cells can bind ET_A-R and possibly ET_B-R expressed by the same cells and therefore exert mitogenic effects through an autocrine system of action. Such effects were also blocked with the addition of BQ123 and ET_A-R antisense [(+): stimulation, (-): inhibition)].

12.2 Endothelins in breast cancer

Results presented in the previous chapters of this thesis have shown that MDA-MB-231, T47D and ZR-75-1 breast cancer cells express mRNA for ET-1 and ET-3 and produce immunoreactive ET-1-like peptide. Regarding the expression of ET receptors, RT-PCR and ligand binding experiments have shown that no ET-receptor mRNA or peptide was expressed in these cell lines. Consistent with the absence of ET-receptors, exogenous addition of ET peptides had no effect on the growth of these cells. Therefore, and in comparison to what can be observed in ovarian cancer, no autocrine control of growth can be in place in breast cancer cells.

Breast fibroblast cell lines BRF1 and BRF2 were shown to express mRNA for ET-1 and not for ET-2 and ET-3, but no ET-1-like protein was detected in the conditioned media of such cells. RT-PCR and ligand binding assays demonstrated the expression of mRNA and protein for ET receptors by breast fibroblasts.

The exogenous addition of ET-1, ET-2 and ET-3 to breast fibroblasts resulted in activation of growth suggesting that in addition to what has already been suggested for ET-1 (by other studies), ET-2 and ET-3 peptides can also stimulate the growth of breast fibroblasts. Consistent with these results, addition of ET-receptor antagonists (BQ123 for ET_A-R and BQ788 for ET_B-R) blocked partially the mitogenic effects of ET peptides. The levels of ET-1-like peptide secreted by the breast cancer cells were comparable to the levels of exogenous ET-1 that produced mitogenic effects on breast fibroblasts consistent with a possible regulation of growth of fibroblasts by cancer cells in breast cancer. Experiments with these two types of cells in co-culture have shown that both cell types grow faster in such environment compared to growth in isolation. The use of specific ET receptor antagonists, showed data consistent with the involvement of ETs in paracrine regulation of growth.

RT-PCR data using primary breast tumour and normal samples suggested the expression of ET-1, ET-2, ET-3 and ET-receptor mRNA and immunohistochemistry data on primary tumour samples suggested the

presence of mostly ET-1 and in some cases ET-2 and ET-3 in epithelial but not stromal areas of the tumours.

Previously reported data have demonstrated the production of ET-1 from breast cancer cell lines and primary tumours and that ET-1 release was higher in tumour compared to normal and borderline breast cells (Kusuhara et al., 1990; Yamashita et al., 1991). The production of ET-1 from breast cancer cell lines has been shown to be modulated by several factors including bombesin, glucocorticoids (Schrey et al., 1992) and IL-6 (Yamashita et al., 1993a). Studies on ET-receptors have suggested that they are expressed in breast fibroblasts but not in epithelial cells (Baley et al., 1990; Patel et al., 1995) while ET-1 has been shown to exert mitogenic effects on breast fibroblasts (Patel et al., 1995).

Results discussed in this study and those reported before are consistent with a paracrine regulation of growth by endothelins secreted by the epithelial cancer cells and having mitogenic effects on breast fibroblasts. A diagrammatic representation of such a model is shown in figure 12.2.

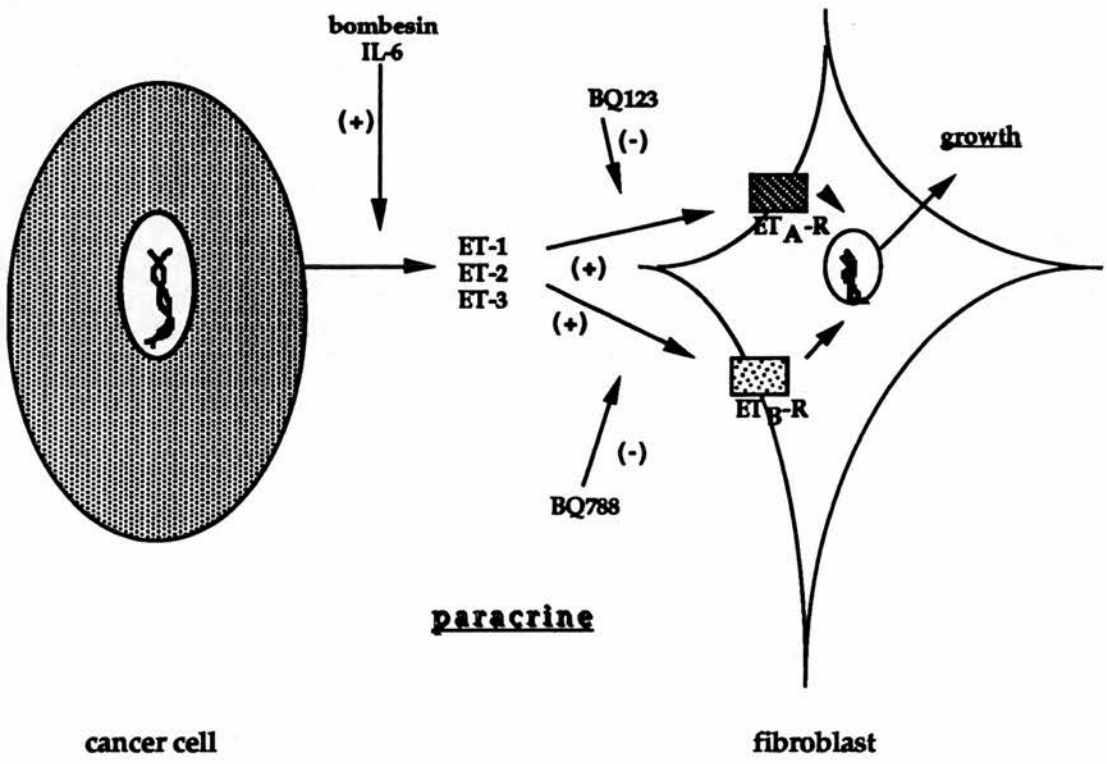


Figure 12.2: A diagrammatic representation of the suggested model of the involvement of ETs on the growth of breast cancer cells and fibroblasts. ETs which are shown to be secreted exclusively by the epithelial cells, can bind to both ET_A-R and ET_B-R ET-receptors on fibroblasts and exert mitogenic effects through a paracrine system of action. Such effects can be blocked using specific ET-receptor antagonists (BQ123 for ET_A-R and BQ788 for ET_B-R). No ET-receptors are expressed in epithelial cancer cells [(+): stimulation, (-): inhibition)].

12.3 Future studies

Experiments described in this thesis provide information regarding only the growth effects of endothelins and the expression and secretion of major components of the mechanisms proposed (release of ETs, ET-receptor expression) due to the restricted time available. Future work on the same theme could investigate the possible differences in the role of each of the three ET peptides and their receptors. The possible interactive role of different peptides and growth factors (insulin, TGF-ß, IGF-I and IGF-II) which may influence the secretion and mitogenic effects of ETs and the signalling mechanisms by which such pathways are activated could be further investigated in both systems following suggestions that some of these factors play a role in the accumulation (TGF-ß), secretion (bombesin, IL-6), metabolism (prostaglandin E_2) and paracrine interaction (IGF-I, IGF-II) of endothelins in other systems (Schrey et al., 1992, 1995; Yamashita et al., 1993). The expression of "big" endothelins and the presence and action of endothelin converting enzyme (ECE) which may regulate the tissue ET levels by processing "big" ETs to active peptides is another area which requires further investigation and has been suggested in breast cancer cell lines (Patel and Schrey., 1995). Further studies could have been focused on the activation of PLC, PLD, PLA_2 and MAP-kinase signalling pathways activated by ETs and the activation of proto-oncogenes such as c-fos, c-myc and c-jun already suggested by previous studies (Simonson et al., 1993; Tabuchi et al., 1994). Finally, the suggestions of a possible role for ETs in angiogenesis of ovarian and breast tumours and their possible interactions with adhesion molecules could also be studied further.

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